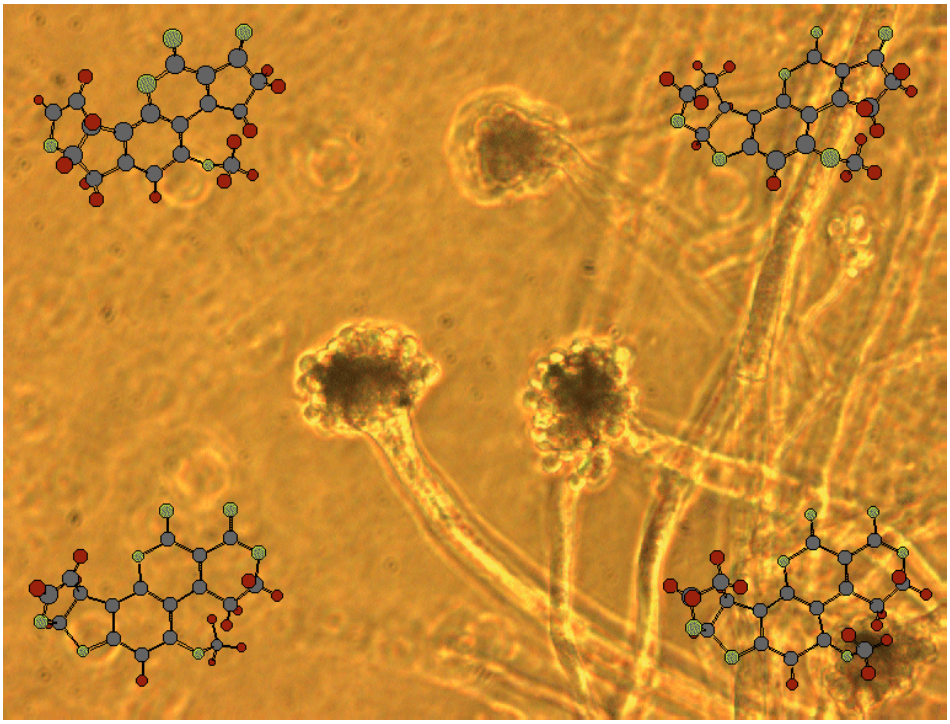
Determination of Aflatoxins in Food and Feed with Simple and Optimised Methods



Jörg Stroka

The following work is based on the guidance of

Prof. Dr. Michael Petz
Bergische Universität-Gesamthochschule Wuppertal,
Department of Food Chemistry

and

Prof. Dr. Elke Anklam
Joint Research Centre of the European Commission
Institute for Health and Consumer Protection
Food Products and Consumer Goods Unit

to whom I would like to address special thanks
for many ideas, helpful hints and support during this work.

June 1997 - Summer 2000

For my mother and for Claudia,

who supported me during all the time

Special Thanks go to the following persons:

Prof. Dr. Gilbert and Dr. Tony Buckle (CSL, York, United Kingdom) for the co-ordination of the SMT-project and Ir. Hans van Egmond (RIVM, Bilthoven, The Nederlands) for hosting the workshops.

Dr. Achim Boenke (DG-Research, Belgium) for the his advice and constructive criticism concerning the deliverables of the SMT-project.

Prof. Dr. Franz Ulberth (Agricultural University Vienna, Austria) for his help and criticism, as well as Mr. Ralf Seyfried (DG-JRC Ispra, Italy) for the help in the construction of the SeBaDeC.

Dr. Urban Joerissen, Mr. Axel Pieper and Mrs. Gunda Niedwetzki (WEJ-Laboratories, Hamburg, Germany) and Dr. Matthias Reuter (LUVA, Kiel, Germany) for the procurement of the homogeneous materials.

Dr. Thomas Peschel and Günther Tittelbach (Fraunhofer Institute for Optics, Jena, Germany) for the tests and the close collaboration during the development of the modified office scanner; Mr. Kobus, Mr. Theissen and Mr. Cornellisen (DG-JRC Ispra, Italy) for the rapid translation of the ideas for the SeBaDeC approach.

Mr. Schmitt (SCHOTT-Mainz, Germany) for the free optical filter samples needed for the SeBaDeC.

Mr. Thomas Rudolph, Mrs. Lisbeth Meyer and Dr. Markus Lipp (DG-JRC Ispra, Italy) for their support during the thesis.

Dr. Christoph von-Holst (DG-JRC-Ispra, Italy) and Dr. Ken Matthison (CSL, York, United Kingdom) for their help in the statistical evaluation and interpretation.

Mr. Robert von Otterdijk for the close collaboration during the development of the TLC-method.

Based on this work, the following publications were put into writing:

1. Stroka J, Joerissen U, Petz M, Anklam E (1999) Investigation of various extractants for the analysis of aflatoxin B1 in different food and feed matrices, Food Addit and Contam 16: 331-338.

2. Stroka J, Anklam E, Joerissen U, Gilbert J (2000) Immunoaffinity cleanup with liquid chromatography using post-column bromination for determination of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study, JAOAC International 83: 320-340

3. Stroka J, Anklam E, Joerissen U, Gilbert, J (2000) Immunoaffinity cleanup with liquid chromatography using post-column bromination for determination of aflatoxin B_1 in infant formula: collaborative study. JAOAC International *in print*.

4. Stroka J, Anklam E, Reutter M (2000) Immunoaffinity cleanup with liquid chromatography using post-column bromination for determination of aflatoxin B_1 in animal feed: collaborative study. JAOAC International *submitted*.

5. Stroka J, Petz M, Anklam E (2000) Analytical methods for the determination of aflatoxins in various food matrices at concentrations regarding the limits set in European Regulations: Development, characteristics, limits, Mycotoxin Research 16: 23-42.

6. Stroka J, van Otterdijk R, Anklam E (2000) Immunoaffinity cleanup prior thin-layer chromatography (TLC) for the determination of aflatoxins in various food matrices. J Chrom A 904: 251-256.

7. Stroka J, Anklam E (2000) Development of a simplified densitometer for the determination of aflatoxins by thin layer chromatography. J Chrom A 904: 263-268.

Abstract

State-of-the-art analytical methods for the determination of aflatoxins in paprika, peanut butter, pistachio paste, fig paste, infant formula and animal feed were developed. All methods employ immunoaffinity cleanup steps, followed by either high-performance liquid chromatography (HPLC) or thin-layer chromatography (TLC). Each method was tested for its suitability to determine aflatoxins in all relevant matrices with focus on method robustness, simplicity of laboratory procedures, toxicity of materials used, and user friendliness. Matrix specific extraction procedures, optimization of the chromatographic separation parameters and derivatization techniques were elaborated for this purpose. Most of the methods were statistically validated in collaborative trials at current legislative limits for aflatoxins and are in the process of adoption as official methods by the European Standardization Committee (CEN) and the Association of Official Analytical Chemists International (AOAC Int.). In addition novel and alternative TLC-densitometer prototypes were developed, and tested in-house. These devices are characterised by their simple construction and low production costs compared to commercial densitometers. The devices were found to be suitable to determine aflatoxins at current legislative levels in combination with adequate TLC methods.

Zusammenfassung

Die vorliegene Arbeit befasst sich mit der Entwicklung analytischer Methoden zur Bestimmung von Aflatoxinen in Paprika, Erdnussbutter, Pistazienpaste, Feigenpaste, Säuglingstrockennahrung und Tierfutter. Grundlage der entwickelten Methoden ist in allen Fällen eine immunchemische Aufreinigung der Probenextrakte, gefolgt von einer flüssigkeits- oder dünnschichchromatographischen Trennung der Aflatoxine. Jede der Methoden wurde auf ihre Anwendbarkeit bezüglich der unterschiedlichen Probenmatrices, der Berücksichtigung von einfachen und robusten Arbeitsschritten, sowie der Giftigkeit der verwendeten Chemikalien und Benutzerfreundlichkeit hin untersucht. Zu diesem Zweck wurden die Extraktionseigenschaften von Extraktionsmitteln bezueglich verschiedenster Probenamtrices untersucht und die chromatographischen Trennparameter sowie Derivatisierungstechniken optimiert. Der überwiegende Teil der Methoden wurde auf dem Niveau geltender Grenzwerte in Ringversuchen validiert und liegt beim European Standardization Committee (CEN) sowie der Association of Official Analytical Chemists International (AOAC Int.) zur Übernahme als Referenzmethoden vor.

Zusätzlich wurden einfache Geräte zur densitometrischen Bestimmung von Aflatoxinen entwickelt und und validert. Diese Geräte zeichnen sich durch ihren einfachen Aufbau gegenüber kommerziellen Alternativen aus. Es konnte gezeigt werden, dass mit den entwickelten Geräten Aflatoxine im Bereich derzeit gültiger Grenzwerte (Europa) sicher bestimmt werden können.

Abbreviations

ADC	Analogue Digital Converter
AOAC	Association of Official Analytical Chemists
Af	Aflatoxin
CAS	Commercially Available Scanner
CCD	Charge Coupled Device
CEN	European Standardisation Committee
df	degree of freedom
DMM	Digital Multimeter
ELISA	Enzyme Linked Immuno Sorbent Assay
GaP	Gallium-Phosphorus
HMF	Hydroxymethylfurfural
HPLC	High Performance Liquid Chromatography
IAC	Immunoaffinity Column
IC	Integrated Circuit
JRC	Joint Research Centre
LC	Liquid Chromatography
LED	Light Emitting Diode
LOD	Limit of Detection
LOQ	Limit of Quantification
MeCN	Acetonitrile
MeOH	Methanol
MoFBeS	Modified Flat-bed Scanner
MS	Mean Squares = SS/df
OA	Operational Amplifier
OA	Operational Amplifier
PBPB	Pyridinium bromide perbromide
PC	Personal Computer
PCD	Post Column Derivatization
RGB	Red-Green-Blue
RP	Reversed Phase
RSD	Relative Standard Deviation
SeBaDeC	Semiconductor Based Densitometer Cell
SS	Sum of Squares
STDEV	Standard Deviation
t-BME	*tert*-Butyl-methyl-ether
TFA	Tri-fluoro-acetic Acid
TLC	Thin-layer Chromatography
UV	Ultra-Violett
VIS	Visible (Light)

Contents

Chapter 1

Introduction and Scope of the Work

1.1 Introduction

The fungi and their toxins Mycotoxins are secondary metabolites which are produced by a number of different fungi. The term *secondary metabolites* was introduced into microbial biochemistry in order to differentiate those compounds such as alkaloids, terpenes, flavanoids and other plant products that may be considered as non-essential for the growth of the plants themselfs. Conversely, amino acids, fatty acids, saccharides, nucleic acids and proteins - compounds essential for all living organisms - were termed *primary metabolites* [1].

Mycotoxins have the following characteristics: (I.) They have a restricted distribution in micro-organisms. (II.) They are characteristic of individual genera, species or strains. (III.) They are formed along specialized pathways from a few primary metabolites such as acetyl co-enzyme A (aflatoxins), mevalonic acid lactone, α-amino acids, and intermediates of the shikimic-acid pathway [2].

Currently there are about 300 different known mycotoxins [3], which are produced by about 200 different fungi of the group of the deuteromycetes (*fungi imperfecti*), which lack a sexual stage of development and propagate vegetatively through asexual spores (conidiospores or conidia) or the vegetative cells.

However, only a small fraction of approximately 20 out of these 300 known mycotoxins are normally found in food and feed at levels that are considered a health risk for humans and animals. Among these, the aflatoxins represent - due to their occurrence and toxicity - the main threat in this field

worldwide. Other commonly known and health relevant mycotoxins are the fumonisins, ochratoxin A, the trichotheceens (e.g. deoxynivalenol, zearaleonone), patulin, cyclopiazonic acid and sterigmatocystin.

Mycotoxins are composed of a diverse range of chemical structures, while in some cases common substructures such as furanofuran- , lactone- , indol- or chinone-elements can be found. These diverse chemical properties result in various toxic effects. Therefore mycotoxins are also classified into groups of hepatoxins (e.g. aflatoxins), nephrotoxins (e.g. ochratoxin A), neurotoxins (e.g. ergot alkaloids) or endocrine disruptors which mimic mammalian hormones (e.g. zearalenon).

Except for the ergot alkaloides, which are produced by *Claviceps purpurea* on grains such as rye, all other relevant mycotoxins that are associated with food or feed contamination are derived from the group of the genera *Aspergillus*, *Penicillium*, *Fusarium* or *Alternaria*.

Historical background One of the first reports in history of mycotoxicosis are related to ergotism, which is caused by the fungi *Claviceps purpurea* or *C. paspali* and can be traced back to ancient times. These fungi infect grain (rye) in the pre-harvest stage and were the cause of severe neurological symptoms as well as many deaths. In the middle ages these symptoms were known as *St. Antony's fire*, while the link between the cause and the symptoms (or deaths) was not known.

Nowadays ergotism is, except for some minor incidences, of neglectable importance for consumer protection because modern food technology allows sufficient ways to control and prevent ergotism. However the problem of mycotoxicosis has not faded. Acute mycotoxicosis is still of concern in animal health, while the human health risks shifted to sub-acute and chronic exposures with long term effects such as suppression of the immuno system, endocrine disfunction and cancer.

The discovery of aflatoxins Aflatoxins are produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. However, other fungi such as *Aspergillus nomius* and *Aspergillus tamarii* have also been reported as aflatoxin producers while they seem to have no significant impact on food contamination.

Aflatoxins were discovered in the early 1960s, when the cause of the so-called *Turkey-X-disease* was identified. This disease resulted in the death of

more than 100,000 turkeys in England during 1960 and was caused by contaminated peanut meal that was fed to the animals. The main responsible toxic metabolites were identified as aflatoxins B_1, B_2, G_1 and G_2, with aflatoxin B_1 (AfB_1) being the most abundant and toxic metabolite in this group. However recently cyclopiazonic acid (CPA) is also discussed as an cause of the Turkey-X-disease in 1960, since *A. flavus* also produces CPA [4].

The name *aflatoxin* is derived from (**A**spergillus **fla**vus **toxin**), since they were first isolated from this species. The classification of the indices B and G is not structure-related and has its origin in the colour of the fluorescence under UV-light (B = blue and G = green), while the structural differences of the terminal furan ring determine the numerical index. Figure 1.1 shows the structures of the four main aflatoxins.

Several other aflatoxin derivatives have been identified in the past. Parasiticol (AfB_3) was found to be another naturally produced metabolite of *A. parasiticus* in 1970, while other derivatives such as AfM_1, AfP_1, AfQ_1, AfD_1 were found as metabolites of other organisms or from chemical degradation [3].

It has been discovered that each aflatoxin producing *Aspergillus* strains is able to produce different and characteristic patterns of aflatoxins. *A. flavus*

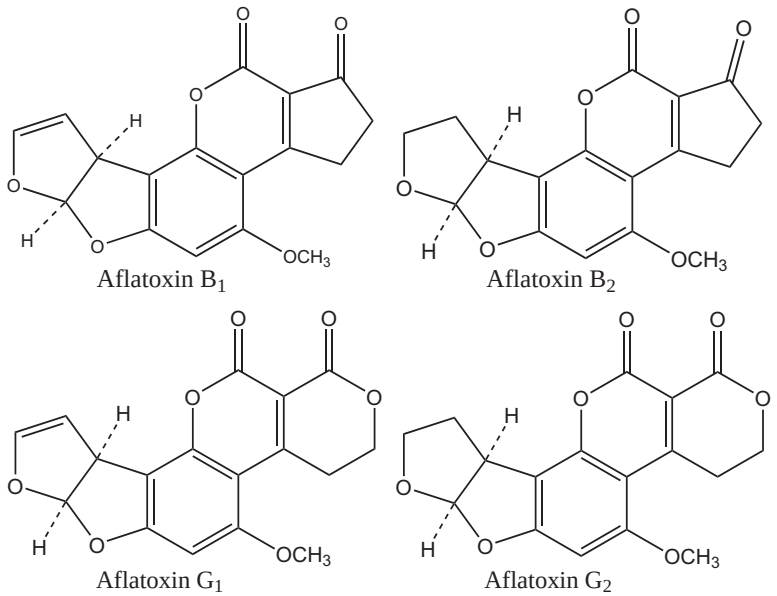


Figure 1.1: Chemical structure of the aflatoxins

produces exclusively AfB_1 and AfB_2, while *A. parasiticus* is able to synthesize all four major aflatoxins (AfB_1, AfB_2, AfG_1 and AfG_2) [5], with AfB_1 and AfG_1 being the major metabolites [6].

These toxin production patterns of the different fungi are reflected in the aflatoxin contamination commonly found to be typical for certain food stuffs. Thus in peanuts all four aflatoxins are commonly detected, since *A. parasiticus* is well adapted to a soil environment. Other food products, derived from aeral parts of plants (e.g. corn, cottonseed, tree nuts) have a pattern typical for *A. flavus*. As a result, over 90% of the contaminated corn samples only contain AfB_1 and AfB_2, while in most contaminated peanut products all four aflatoxins are found [5].

Chemistry of the aflatoxins Aflatoxins are di-furano coumarins with AfB_2 and AfG_2 being the hydrated derivatives of AfB_1 and AfG_1. The biosynthesis pathway of aflatoxins has been postulated as followed: norsolorinic acid → averufin → versiconalacetate → versicolorin A → sterigmatocystin → aflatoxin B_1 → aflatoxin G_1. Figure 1.2 shows the postulated pathway [2].

The toxicity of the aflatoxins decreases from AfB_1 → AfG_1 → AfB_2 → AfG_2, which is an indicator that the double bond at the 8,9-position at the terminal furano ring is a crucial factor for the toxicity of the toxin.

Aflatoxins are heat stable compounds and normally do not degrade during normal food or feed processing. However, several approaches for detoxification have been proposed, ranging from microbial, physical (*extraction, absorption or elevated heat*) chemical or even radiation approaches [7–10] .

Metabolism of aflatoxins Aflatoxins are strong hepatotoxins and are internationally classified as carcinogens [11]. All animal species tested are susceptible to aflatoxins with ducklings being most sensitive [LD_{50}= 0.4 μg/kg] [9]. The metabolic pathway of the aflatoxins is still not fully understood, while it is generally accepted that the metabolism of aflatoxin B_1 consists of two stages. First it is converted by cytochrome P-450-mediated mixed-function oxidase into active electrophilic intermediates (Figure 1.3) and then conjugated covalently with nucelophiles such as DNA (Figure 1.4), RNA or proteins in the liver cells [9, 12]. The formation of highly reactive 8,9-epoxide intermediates has been first postulated and then confirmed experimentally. It has further been postulated that this electophil 8,9-epoxide is formed by the microsomal mixed-function mono-oxygenase (MFO), which is the causative agent for the toxicity and/or carcinogenicity [13].

A total of six human metabolites have been found to appear in blood,

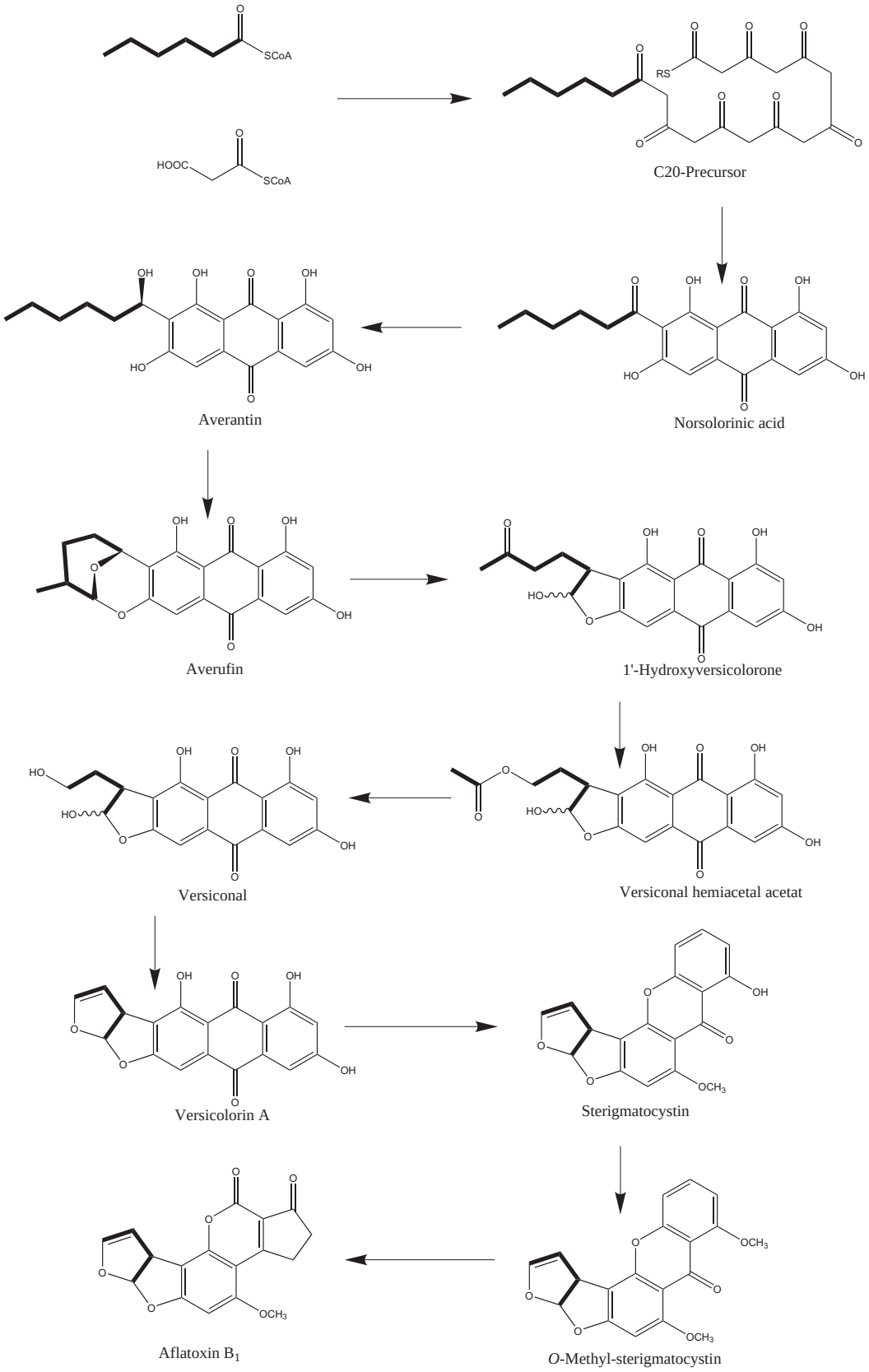


Figure 1.2: Biosynthesis pathway the aflatoxins

urine and tissue such as liver, umbilical cord and milk [9] (Figure 1.3).

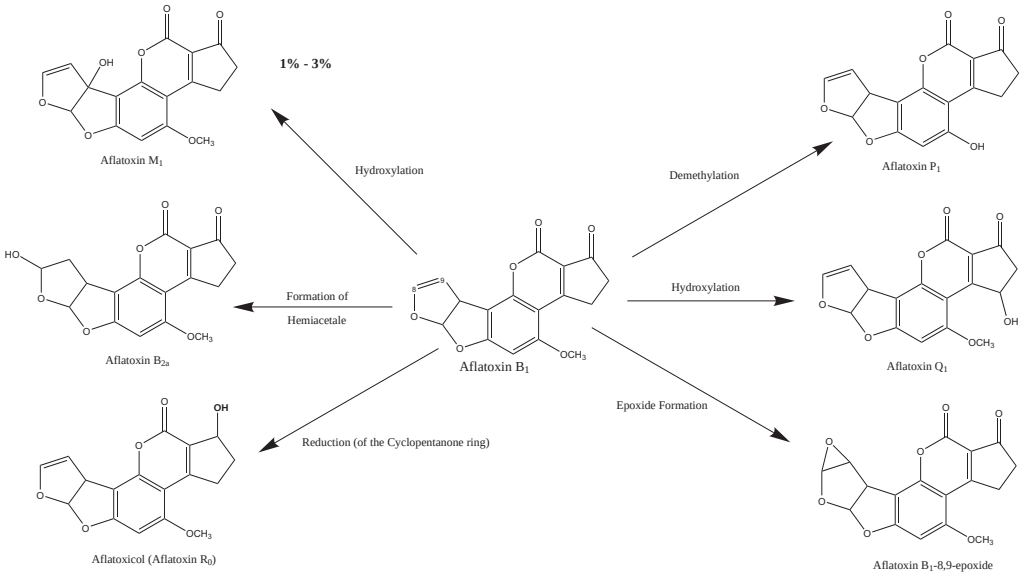


Figure 1.3: Metabolic pathways of aflatoxin B_1

The aflatoxin-guanosine adduct (Figure 1.4) can result in a mutation of the tumour suppressor gen p53. It has been postulated that thymine will be read (or formed) instead of guanosine, which as a result will be followed by a false translation of the coded protein (replacement of arginine with serine), which has been found in liver tumour cells [14, 15].

One of these metabolites is aflatoxin M_1, which is the main metabolite found in cattle milk. Approximately 1% to 3% of the ingested AfB_1 can be found as AfM_1 in the milk of cattle [16, 17]. AfM_1 is less toxic than AfB_1, however due to the high consumption and the importance of milk in human nutrition, this metabolite is routinely monitored.

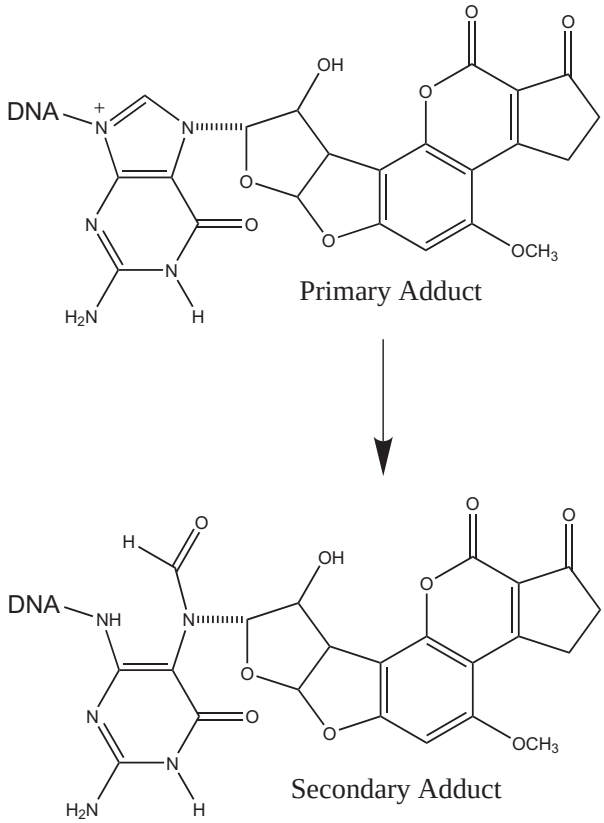


Figure 1.4: DNA-aflatoxin B_1 conjugates

Occurrence of aflatoxins in food and feed The growth of the aflatoxin producing Aspergillus specis depends on several substrate and environmental factors, such as water activity, temperature, pH, redox potential, presence of preservatives and microbial competition. As a result, *A. flavus* and *A. parasiticus* are considered as xerophilic since they can grow at low water activities (a_w 0.75-0.8). Both fungi can grow in a temperature range from 12°C to 48°C, while the optimal growth temperature ranges from 25°C to 42°C [18], with the best conditions for aflatoxin growth in the lower part of this range at approximately 25°C [19].

The produced aflatoxins can be found in a diverse range of products due to non-visible spoilage in the field (pre-harvest), storage or processing (post-harvest).

However, high contamination levels of aflatoxins are mainly associated with post-harvest growth of Aspergillus moulds in poorly stored commodities. Aflatoxin concentrations in the mg/kg range have been detected in such

cases [20, 21].

The most relevant food items that have been reported to contain aflatoxins are cereals such as corn, barley and oats, dried fruits such as figs, nuts and oilseeds such as pistachios or peanuts and cotton seeds as well as spices such as pepper, paprika or chillis [1]. However, corn and peanuts are the most frequent contaminated food items worldwide [22].

Several surveys on the occurrence of aflatoxins have been reviewed [9, 10, 23–25] and clearly show that the occurrence of aflatoxins in food and feed is still a relevant issue in food safety. Table 1.1 gives an overview of recent studies on aflatoxins in food stuffs [24].

Commodity	Country	Publ. Year	No. of Smp	Incidence (in %)	Range (μg/kg)	Ref.
Corn	India	1997	2074	47	5 - 666*	[26]
Corn	Venezuela	2000	37	14	5 - 50*	[27]
Corn	Argentinia	1996	2271	20	5 - 560*	[28]
Peanuts	India	1996	2064	45	5 - 833*	[29]
Peanut meal	India	1995	380	97	8 - 6280*	[30]
Cottonseed	UK	1997	21	71	5 - 25	[31]
Copra meal	Philippines	1995	9	100	23 - 186*	[32]
Brazil nuts	USA	1993	176	17	trace - 619	[33]
Various nuts	Qatar	2000	81	23	0.53 - 289	[34]
Pistachios	Qatar	2000	101	48	1.2 - 274	[34]
Pistachios	NL	1996	29	59	2 - 165*	[35]
Almonds	USA	1993	44	1	trace - 372	[33]
Soybeans	Argentinia	1991	94	10	1 - 36	[36]
Rice	Ecuador	1997	99	9	6.8 - 40*	[37]
Wheat	Uruguay	1996	123	20	2 - 20	[38]
Dried figs	Austria	1993	136	13	1 - 350	[39]
Nugmet	Japan	1993	67	43	0.2 - 666	[40]
Chillies	Pakistan	1995	176	66	1 - 79.9*	[41]
Ethnic foods	UK	1996	121	61	0.1 - 61	[42]

Table 1.1: Occurrence of aflatoxins in food
(*aflatoxin B_1 only)

Epidemiological studies Several epidemiological studies have been carried out to determine the intake and the concentration of aflatoxins in the human body [9, 17, 43].

It was shown that aflatoxins can be found in significant fractions of different populations. Studies carried out in Africa indicated that approximately 12% - 37% of the African population has measurable amounts of aflatoxin in the blood serum [43]. The daily intake of aflatoxins was estimated to be 2.7 ng per kg body weight per day for US citizens, 3.5 to 55 ng/kg bw/day for Thai and up to 220 ng/kg bw/day for Africans. However certain authors reported intake estimations of up to 22000 μg (absolute) per day [9, 22].

Correlations between aflatoxin intake and certain diseases were found, while links to primary liver cancer, Reye's syndrome, Kwashiorkor and other malignant diseases are currently discussed [9, 17, 22, 44].

Mycotoxin regulations Due to the potential health risk of mycotoxins for humans and animals, legal limits for food and feeding stuffs have been established in at least 77 countries worldwide with different levels ranging from 0 μg/kg to 50 μg/kg for aflatoxin B_1 or total aflatoxins [45]. For animal feed at least 75 countries have introduced or proposed regulations for aflatoxin control, while aflatoxin M_1, as the main metabolite of aflatoxin B_1 in dairy products, is regulated in at least 22 countries [16] at levels from 0 μg/kg to 1 μg/kg.

To assure proper consumer protection, the European Commission recently established legal limits in the lower μg/kg range (2 μg/kg aflatoxin B_1 and 4 μg/kg total aflatoxins) for food and for infant formula at 0.1 μg/kg aflatoxin B_1 [46]. In addition to these regulations, the European Commission recognized the relevance of aflatoxin exposure for human health by adopting regulations that banned the import of certain food stuffs for a limited time [47, 48].

In addition to these health concerns for humans and animals, economical reasons are another drive for regulations. As an example, due to estimated aflatoxin levels in feed, a weight reduction of approximately 3% was calculated for US broilers, which was found to be equivalent to a loss of 140 million US$ per year [49], while the annual costs for the detoxification of aflatoxin contaminated peanuts and the post harvest losses in Australia, Indonesia and the US are estimated to 3 to 6 million Australian $, respectively 90 million A$ for Indonesia and 210 million A$ for the USA [50].

Methods of analysis Methods of analysis for aflatoxins have undergone continuous development since the aflatoxins were discovered in the early

1960s. This development was mainly due to legislative changes as well as to the continuous progress in analytical chemistry.

As a result, the exposure of humans and animals to mycotoxins was mainly limited through chemical screening/monitoring programmes of the suspected commodities [1]. These programmes, however, depend directly on *precise* and *reliable* analytical methods for mycotoxin determination in sometimes rather complex food and feed matrices.

Therefore requirements for analytical methods have been established at national and international level. Internationally relevant requirements are laid down by organizations such as the European Committee for Standardization (CEN) or the Association of Official Analytical Chemists International (AOAC International). For adoption as *official methods* any proposed method should be validated in a collaborative trial study. Minimum method performance characteristics [51], the framework for the conduct of collaborative trial studies as well as the statistical evaluation are clearly defined in protocols for the adoption [52]. Any method that has been established and tested according to these protocols can be recognized as *official method* for use in legal cases or for international trade.

Several analytical method principles for the determination of aflatoxins have been developed, ranging from thin-layer chromatography (TLC), high performance liquid chromatography (HPLC), fluorimetry or enzyme linked immuno sorbent assay (ELISA) techniques [1, 53–57].

Nowadays, economical aspects become more important in method development. Particularly fast and efficient procedures (material and chemicals consumption) as well as automatization [58] are highly desired features.

Even though several methods have already been validated in collaborative studies with some success, there was still a need for further method development and validation. Previously validated method were mainly limited to matrices such as peanuts or corn, while for food products such as figs, pistachios, paprika or infant formula no internationally recognized method was available for the desired purpose here. Particularly the target contamination range of aflatoxins of already available methods [59] was significantly higher compared to the recently required target level of 1 μg/kg to 5 μg/kg for aflatoxins [46].

One reason that no method was previously validated at an international level for the targeted contamination range was thought to be due to the predicted poor precision data that would be obtained in such a collaborative trial study [60].

However, recent progress in analytical chemistry, such as the availability of immunoaffinity columns for aflatoxins [61] and new post-column derivatization techniques for HPLC [62] were found to improve analytical results

significantly [63].

It was shown that with care and attention to detail during organization of a collaborative trial impressive performance characteristics can be obtained, even with classical solid phase sample preparation methods in combination with HPLC [64].

A UK collaborative trial with peanut butter was carried out when immunoaffinity columns first became available (1989) which initially indicated promising results [65]. With a subsequent international collaborative trial in 1990 [80], using post-column derivatization with iodine, some participants experienced problems with recoveries and despite acceptable precision data the method was not pursued for AOAC recognition.

Another collaborative trial [67] organized in the UK for food control analysts (21 public analyst laboratories) again for peanut butter reported RSD_r-values ranging from 17% to 44% and RSD_R-values ranging from 36% to 54% for concentrations of total aflatoxins from 7 μg/kg to 47 μg/kg. The precision parameters were regarded as rather high for this trial and the samples distributed were atypical having aflatoxin G1 as the principal aflatoxin in the mixture of aflatoxins in the peanut butter.

The most successful collaborative trial was carried out in 1990 [68], based on immunoaffinity column clean-up of samples and either solution fluorimetry or post-column derivatization with iodine for determining total aflatoxins in corn, peanuts and peanut butter. This method was adopted by AOAC International [59].

Due to the legislative limits for aflatoxins in food in the lower μg/kg range, the European Commission recognized the need for an adequate analytical method and the recognition by CEN through validation [69]. The desired methods should have performance characteristics similar or better than the above discussed methods, while the analytical procedures should be simple and rapid enough to make use of the method for routine work.

1.2 Scopes of Interest

The aim of this work was the development of state-of-the-art analytical HPLC and TLC methods for further validation in collaborative trial studies.

Current European legislative levels for food and feed matrices in addition to simplicity and robustness of the method were the main goals in this work, since these are crucial aspects in modern analytical chemistry [70].

Since HPLC and TLC approaches both have their assets [19], it was decided to initially further improve existing methods for TLC and HPLC. In a second step, these methods were foreseen to be validated through collabor-

ative trial studies, with the aim of adoption as official methods by AOAC International and CEN.

Chapter 2

Results and Discussion

2.1 Method Development for a HPLC Method

2.1.1 Sample Extraction

Interaction between solvent and sample (*Salting Out Effects and Water Adsorption*) Prior to detection and quantification of the aflatoxins, they must be isolated from the rather complex and variable sample matrices. This is generally done by extraction of the sample material with an organic solvent, in order to dissolve the aflatoxins for further clean-up.

Several extractants have been proposed for the extraction of aflatoxins from food and animal feeds [71] [72–76]. Since modern clean-up procedures for aflatoxins are based on immunoaffinity [1, 61], the extractants used for extraction consist preferably of an aqueous organic solvent such as methanol (MeOH), acetonitrile (MeCN) or acetone [1, 61, 77–79] [80, 81] [82–84].

Organic solvents other than these are less favorable, since they cannot be applied directly onto the IAC and require the removal of the solvent, while diluted aqueous extractants are easy to use for automated immunoaffinity clean-up procedures with subsequent HPLC determination of the aflatoxins [85]. Automatisation was found to improve recoveries and repeatability [61].

Several aqueous extractants of MeOH, MeCN and acetone as well as various extractant-to-sample-ratios have been under investigation and were compared for recovery in various sample materials [77, 86, 87]. However, none of these publications considered possible matrix-extractant interactions and resulting composition changes of the filtered extracts, while the focus of these studies was on the recovery of the analyte.

In order to study possible effects and interactions of soluble matrix constituents with the extractant, sodium chloride or sucrose were added directly to those aqueous extractants that were known to be commonly used for aflatoxin B_1 determination. No phase separation occurred in the aqueous MeOH extractants (6+4 and 8+2), while from the extractants containing MeCN (6+4) or acetone (6+4 and 8+2) a layer separation occurred when sodium chloride was added. In addition only the aqueous MeCN (6+4) extractant resulted in a layer separation by adding sucrose. The results of the salting out experiments are listed in Table 2.1.

Extractant [100 ml]	**NaCl added [in g]**	**Effect observed**	**Sucrose added [in g]**	**Effect observed**
MeCN+water (6+4)	3.0	Layer separation	5.5	Layer separation
Acetone+water (8+2)	2.5	Layer separation	10	No effect
Acetone+water (6+4)	5.5	Layer separation	10	No effect
MeOH+water (8+2)	4.0 (Saturation)	No effect	10	No effect
MeOH+water (6+4)	9.0 (Saturation)	No effect	10	No effect

Table 2.1: Layer separation of various extractants by sucrose or sodium chloride (sucrose was added up to an amount of 10 g. Any further addition is not relevant for practical purposes)

In order to investigate the distribution of aflatoxin B_1 in the phase layers of the above-described extractants, experiments were repeated by using spiked extractants. The results listed in Table 2.2 show that aflatoxin B_1 is not equally distributed between the layers of all separated extractants.

Extractant (50 ml)	**AfB_1 in upper layer [in %]**	**AfB_1 in lower layer [in %]**
MeCN+water 6+4 (2 g NaCl)	93	7
MeCN+water 6+4 (3 g Sucrose)	93	7
Acetone+water 8+2 (1.5 g NaCl)	88	12
Acetone+water 6+4 (3 g NaCl)	69	31

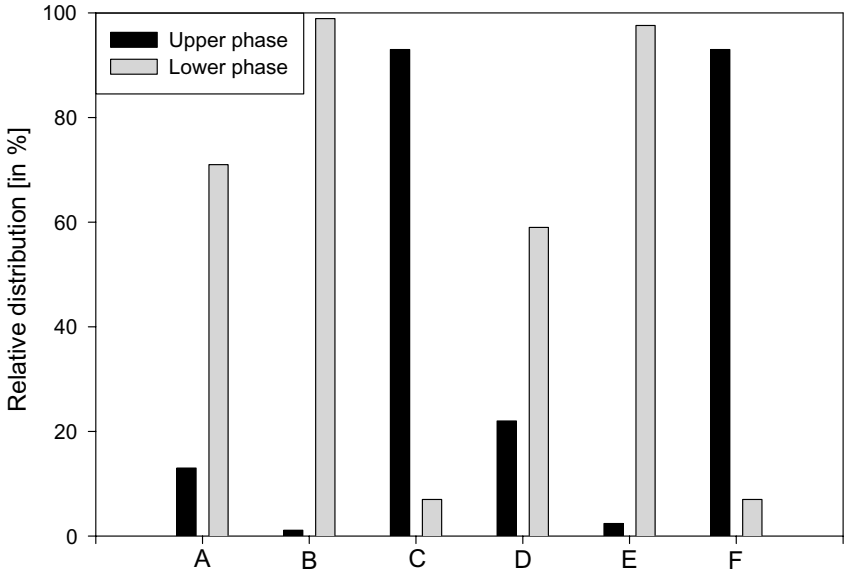
Table 2.2: Distribution of AfB_1 in the extractant layers after separation

Furthermore, the contents of water and of dry mass (residues) of separated phases were determined. It could be shown that the compositions of the layers regarding the water content were significantly different (17% water in the upper phase and 71% water in the lower phase for MeCN-water (6+4) and 24% water in upper phase and 53% water in the lower phase for acetone-water (8+2), while the water content of the original solvents were 46.5% for MeCN-water respectively 23.5% for acetone-water). Figure 2.1 shows the distribution of all relevant parameters (aflatoxin B_1, the dry mass and water content) for MeCN+water (6+4).

As can be seen, soluble constituents of a food matrix can interfere with the extractant and alter analytical results. In fact further experiments with fig-paste (representing a food matrix with a high content of soluble sugars) revealed that fig extracts from MeCN do separate after filtration occasionally and that the resulting layers are often difficult to identify. As a consequence any quantitative results obtained from such extracts, is of doubt.

Furthermore, it could be shown that the compositions of the separated layers regarding the water content were significantly different. MeCN-water (6+4) separated to an upper solvent phases with 17% water content and another one with a water content of 71%. Acetone-water separated to a system with 24% water in upper phase and 53% of water in the lower layer, while the water content of the original solvents were 46.5% for MeCN-water (6+4 [v/v]) respectively 23.5% for acetone-water (8+2 [v/v])).

These results clearly show that the liquid constituents (water and organic solvent) do not separate completely, while the composition difference is significant enough for the aflatoxins and other soluble components to dissolve almost fully in only one solvent layer.



Results of the determination of components in the separated phases after addition of sodium chloride (A - C) or sucrose (D - F). Columns A and D reflect the water content, columns B and E the residues after evaporation (dry mass) and columns C and F the aflatoxin B_1 distribution.

Figure 2.1: Distribution of extractant constituents after layer separation

A visible phase layer separation of MeCN and acetone extracts as they occurred with figs were not observed for matrices such as paprika powder, animal feed or infant formula. However it was observed that the volume of filtrate decreased significantly for MeCN extracts, depending on the amount of sample used for extraction.

In addition certain extracted sample materials clotted during extraction with MeCN-water (6+4), while extracts derived from MeOH-water (8+2) and acetone-water (8+2) resulted in a distinct separation of the extractant from the deposited matrix particles (Figures 2.2 and 2.3). This effect can be traced back as water adsorption by the matrix in addition to a *non visible* phase separation, which will lead to the same effect as the observed salting out.

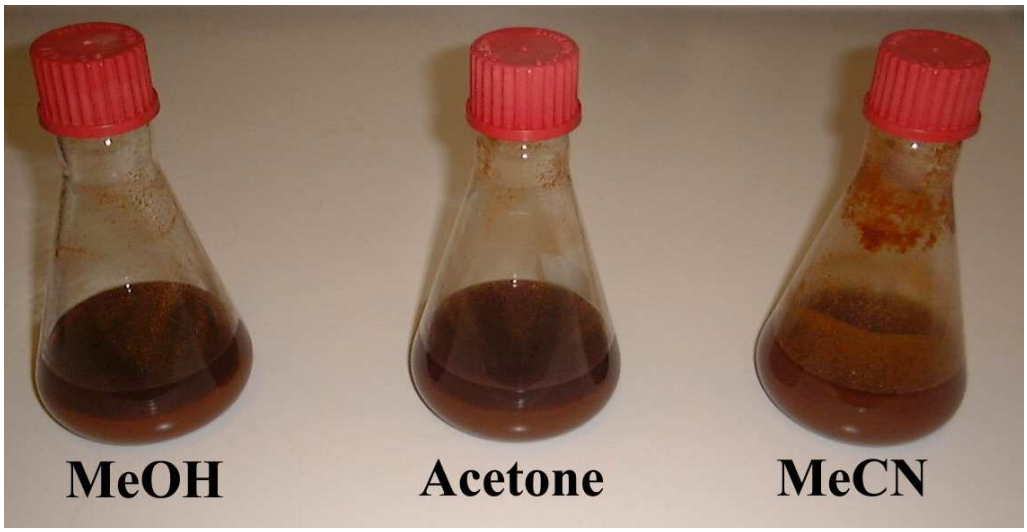


Figure 2.2: Difference in the sedimentation of paprika powder with different extractants

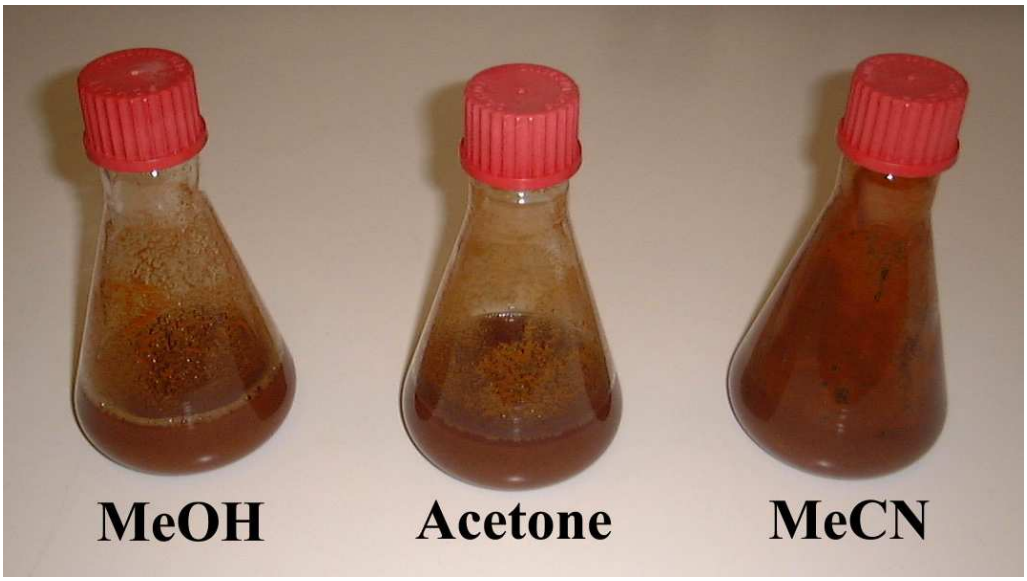


Figure 2.3: Clotting of paprika powder
in aqueous MeCN

For further elucidation of these effects, various dry sample materials were extracted with different extractants. The water content in the filtered extracts was subsequently determined by the Karl-Fischer method and compared with the water content of the original extractant. The observed differences in the water content were then plotted against the content of organic solvent in the original extractant. The results are given in Table 2.3 and 2.4. Figures 2.4, 2.5 and 2.6 show the effect of the water absorption depending on the extractant used. As can be seen, the reduction of the water content in the final extracts varied significantly, depending on the origin of the organic solvent in the extractant, the composition of the mixture, and the matrix.

Sample material:	**gram sample pro 100 mL**	**MeCN:H_2O (6+4 [v/v])**	**MeOH:H_2O (8+2 [v/v])**	**Acetone:H_2O (8+2 [v/v])**
Soy based infant formula	10	3.9	1.4	1.1
	20	8.4	3.3	3.2
	40	18.3	5.6	5.7
Milk based infant formula	10	2.8	0.3	0.1
	20	6.5	0.9	0.7
	40	18.3	3.0	1.0
Animal feed #1	10	0.9	0.2	0.8
	20	1.8	0.5	1.5
	40	4.6	0.6	5.1
Animal feed #2	10	1.8	0.8	0.7
	20	3.2	0.4	0.7
	40	14.2	1.1	1.8
Animal feed #3	10	3.5	2.0	2.0
	20	10.9	3.1	3.8
	40	23.6	5.0	8.2
Paprika #1	10	1.3	0.5	1.1
	20	6.4	2.3	2.4
	40	21.2	3.7	4.1
Paprika #2	10	2.8	0.9	0.4
	20	6.5	2.3	1.7
	40	20.5	3.4	4.2
Paprika #3	10	2.8	1.2	1.1
	20	7.9	2.3	3.0
	40	24.1	4.9	6.0

Table 2.4: Water loss in the extration filtrate
for different sample-to-extractant ratios

Matrix	**Water in the extractant**			
	50%	**40%**	**30%**	**20%**
$\text{Babyfood}_{\text{Milk}}$ Acetone	3.6	2.3	2.2	0.7
$\text{Babyfood}_{\text{Milk}}$ MeCN	2.8	12.2	8.8	6.5
$\text{Babyfood}_{\text{Milk}}$ MeOH	5.5	4.0	4.3	0.9
$\text{Babyfood}_{\text{Soy}}$ Acetone	6.7	4.3	3.7	3.2
$\text{Babyfood}_{\text{Soy}}$ MeCN	7.6	9.7	9.3	8.4
$\text{Babyfood}_{\text{Soy}}$ MeOH	4.7	4.4	4.5	3.3
Animal feed_1 Acetone	3.9	3.0	1.7	1.6
Animal feed_1 MeCN	3.6	4.5	6.0	1.8
Animal feed_1 MeOH	1.1	1.9	0.9	0.5
Animal feed_2 Acetone	3.3	1.5	0.6	0.7
Animal feed_2 MeCN	3.7	1.0	2.6	3.2
Animal feed_2 MeOH	0.8	0.6	0.6	0.4
Animal feed_3 Acetone	6.1	4.4	3.1	3.8
Animal feed_3 MeCN	6.3	12.7	13.3	10.9
Animal feed_3 MeOH	4.1	3.0	3.2	3.1
Paprika powder_1 Acetone	1.3	1.1	1.0	2.4
Paprika powder_1 MeCN	2.9	6.4	9.4	6.4
Paprika powder_1 MeOH	2.2	0.4	2.6	2.3
Paprika powder_2 Acetone	3.9	3.5	2.4	1.8
Paprika powder_2 MeCN	3.2	8.3	10.4	6.5
Paprika powder_2 MeOH	1.8	1.2	2.6	2.3
Paprika powder_3 Acetone	5.8	3.9	3.4	3.1
Paprika powder_3 MeCN	5.2	13.7	13.4	7.9
Paprika powder_3 MeOH	3.0	2.5	4.6	2.3

Table 2.3: Water loss in the filtrate for different water contents in the extractant

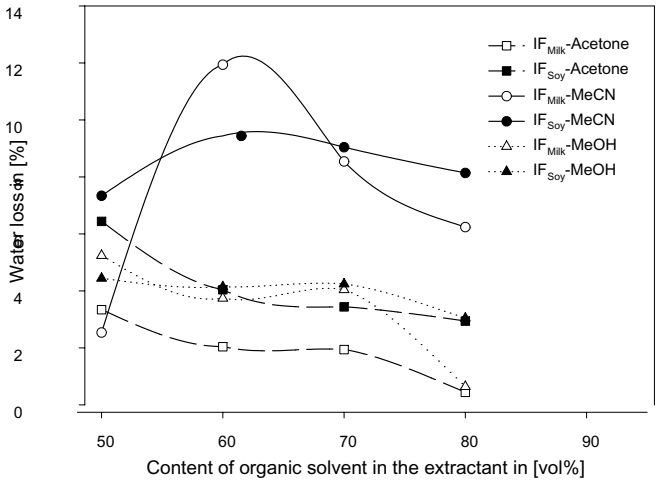


Figure 2.4: Water loss in different baby food matrices

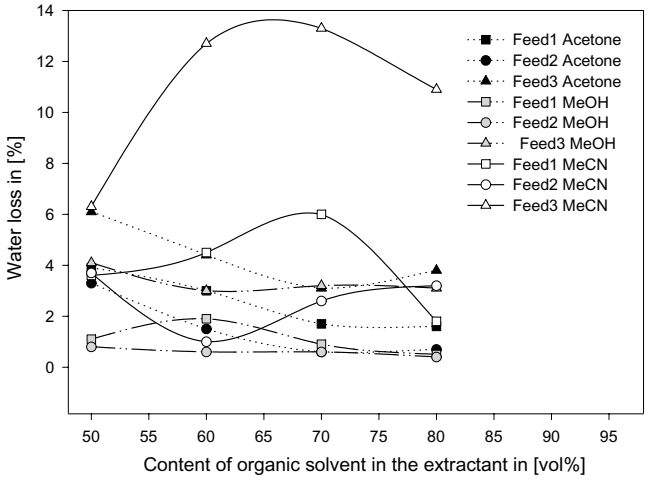


Figure 2.5: Water loss in different animal feedingstuff

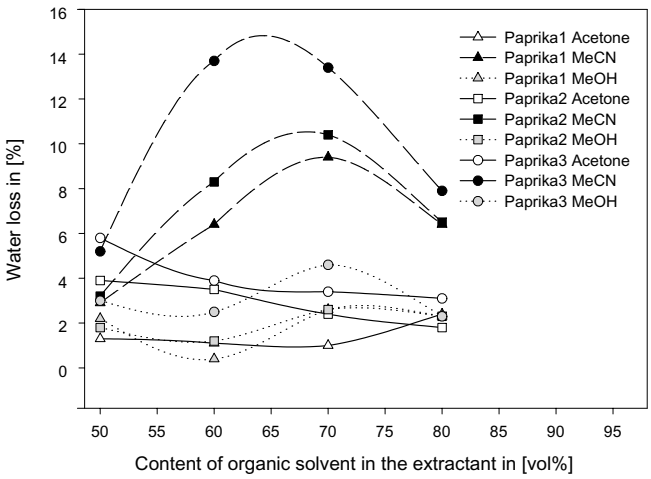


Figure 2.6: Water loss in different paprika powders

The use of aqueous MeCN extractants resulted in significant water losses with maximum water losses between a solvent-to-water ratio of (6+4) and (7+3), while the water loss for extractants based on MeOH or acetone was found to be much smaller and relatively constant over the whole range of solvent-to-water ratios.

These differences regarding the water content were taken as an indicator of the adulteration (formation of a pseudo layer separation) of the extractant during sample extraction. As will be demonstrated later on, such an adulteration leads to false higher recoveries, and explains (even though no direct indication for the choice of MeCN-water (6+4) as extractant was found in the literature) the popularity of this extractant in terms of analyte recovery.

To substantiate these findings and verify that the decreased water contents in the filtrates were only insignificantly influenced by extracted substances from the sample matrices, the dry mass (solvent free residue) of the extracts was determined. The results are listed in Tables 2.5 and 2.6, while Figures 2.7 and 2.8 give a more transparent view on these results.

Sample Material	**MeOH:H_2O** (8+2)	**Acetone:H_2O** (85+15)	**MeCN:H_2O** (6+4)
$\text{Babyfood}_{\text{Milk}}$	2.5	1.8	1.5
$\text{Babyfood}_{\text{Soy}}$	9.0	1.0	2.0
Animal feed_1	2.7	1.3	2.6
Animal feed_2	1.8	1.3	1.9
Animal feed_3	11.2	0.8	1.7
Paprika powder_1	8.7	4.3	3.8
Paprika powder_2	9.9	3.0	3.4
Paprika powder_3	11.7	3.3	2.7

Table 2.5: Dry mass of various sample extracts
(solvent free residue in %) at a ratio of 20 g per 100 mL

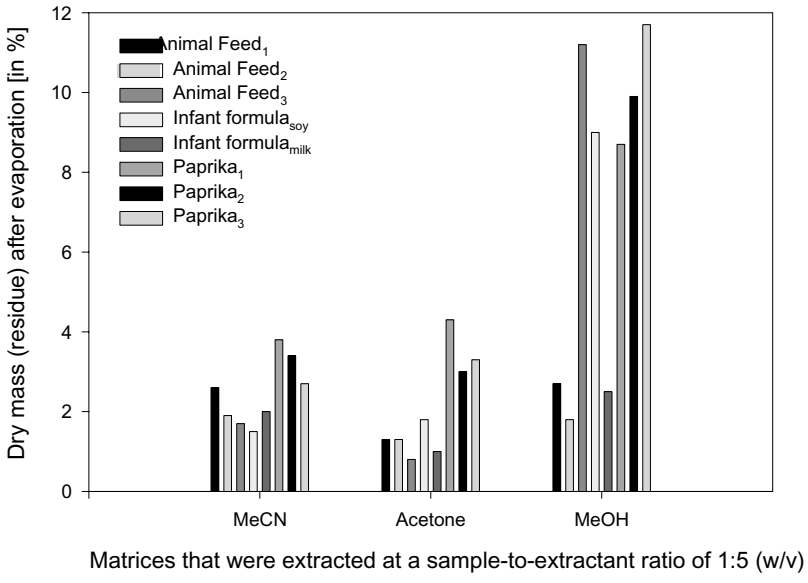


Figure 2.7: Dry mass (residue) depending on the extractant

g/100 mL	Methanol			Acetonitrile			Acetone		
	#1	#2	#3	#1	#2	#3	#1	#2	#3
10	2.0%	2.6%	3.4%	2.5%	2.7%	3.3%	2.3%	2.5%	2.8%
20	8.7%	9.9%	11.7%	3.8%	3.4%	2.7%	4.3%	3.0%	3.3%
30	11.0%	12.9%	14.9%	4.6%	3.0%	3.0%	4.5%	3.2%	3.6%

Table 2.6: Dry mass of different paprika extracts
(solvent free residue in %) for several sample-to-extractant ratios

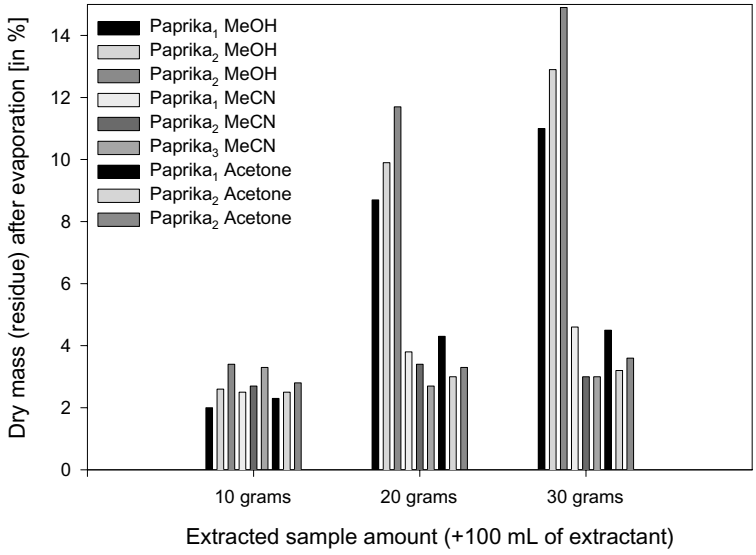


Figure 2.8: Dry mass (residue) of paprika extracts

As can be seen, the amount of soluble substances in the MeCN and acetone extracts are significantly lower for several matrices than in the MeOH extracts, which is due to the more polar nature of MeOH [88]. In the case of paprika it was shown that a saturation of the extract with the main soluble constituents of the sample matrix is already reached at a sample-to-extractant ratio of 10 g per 100 mL when extracted with acetone-water, while these amounts continuously increase in aqueous MeOH extractants up

to a ratio of 40 g per 100 mL, which is the line of practicable procedures.

It must be mentioned, that these *dry mass* experiments must be interpreted with care, at least in the case of aqueous MeCN, since the filtered extracts had a different composition than the original extractants used. Generally in these extracts the water content was decreased and therefore the polarity decreased, while the solubility of the according *solvent layer*, which remained in the filter cake was unknown.

To prove that the described high water losses in the filtered extracts from aqueous MeCN lead to false (higher) analytical results, the aflatoxin B_1 content in naturally contaminated and homogenous paprika material was analysed. Extractions were carried out with an aqueous MeCN, aqueous MeOH and aqueous acetone extractant. The obtained values are listed in Table 2.7.

When these data are plotted into a diagram (Figure 2.9) it can be seen that the determined aflatoxin B_1 content depends strongly on the solvent-to-sample ratio used for analysis, using aqueous MeCN. This clearly indicates that the analytical result is a direct function of analytical parameters, which should have no significant influence in robust procedures. For extractions made with aqueous MeOH or acetone, the solvent-to-sample ratio is less critical in terms of the analytical results for aflatoxin B_1.

Furthermore, aqueous MeOH extracts result in slightly smaller recoveries than those obtained with acetone. A reason for this can be the large amounts of dissolved substances in the extract, which to a certain degree cause a certain dilution of the extractant.

However, if these values are taken to establish a function, for the extrapolation of analytical results that would be analysed and assuming no interaction between sample and extractant (value extrapolated to the y-axis at (x=0)), it can be shown that none of the tested extractants has a significant

Solvent system	**MeOH:H_2O** (8+2 [v/v])	**Acetone:H_2O** (8+2 [v/v])	**MeCN:H_2O** (6+4 [v/v])
10 g Sample/ 100 mL	2.64	3.05	3.99
20 g Sample/ 100 mL	2.78	3.21	4.80
30 g Sample/ 100 mL	2.38	3.12	5.22
40 g Sample/ 100 mL	2.33	3.11	5.44

Table 2.7: Influence of the extractant on the analytical result of aflatoxin B_1

advantage in terms of a better recovery.

To further support the findings that extractions made with aqueous MeCN lead to false recoveries, fortified (spiked) blank paprika material was analysed. Recoveries ranged from 137% to 170% for aqueous MeCN extraction (40 g/100 mL) clearly showing a crucial error in this method, while for lower ratios (10 g/ 100 mL) this effect will be *masked* and the recoveries were found to be in an acceptable range of 78% - 89%. Recoveries obtained with aqueous MeOH were inconspicuous for the whole range of 10 g to 40 g of sample material per 100 mL extractant and varied from 75% - 94%.

These experiments showed that aqueous MeCN is an inadequate extractant for aflatoxin analysis and might lead to false analytical results. Despite its popularity as extractant it should therefore be replaced by either aqueous acetone or MeOH.

Furthermore, it should be stressed that another drawback of MeCN is the unsteady water absorption (*even though only an indicator*) by matrices of the same origin. As already shown in Table 2.3 the water-loss within a

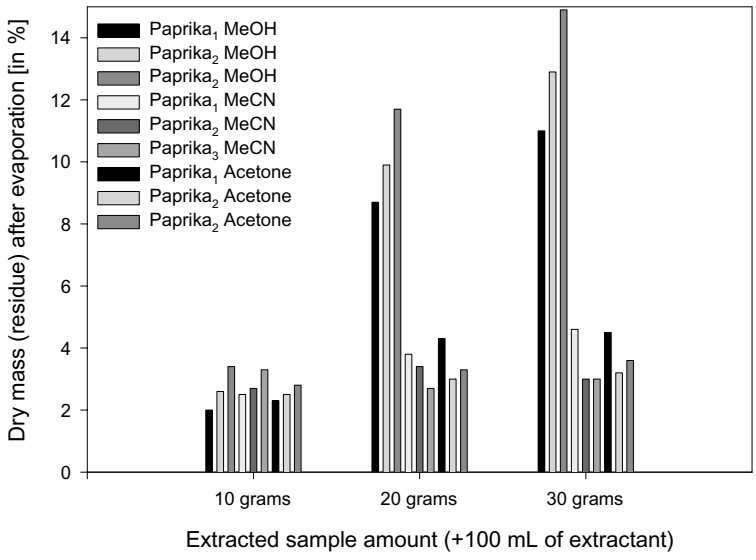


Figure 2.9: Dependency of analytical results on the extractant

very specific food group such as paprika powder might differ significantly (6%, 8%, and 14% for MeCN [6+4]). This fact indicates an unpredictable interaction and thus makes it impossible to correct any results for recovery. Nevertheless the loss of water should not be used for correction of analytical data, since it reveals no information of the volumes of the separated *phases* formed during extraction, thus the analyte concentration.

These effects should not only concern the determination of aflatoxin B_1 but also all those analytes that have comparable solubility and distribution properties in separated solvent layers. Therefore these effects should generally be taken into consideration when extractants are compared or selected for recovery and suitability.

Matrices with a high content of fat (*Pistachio Paste and Peanut Butter*) Peanuts and pistachios are matrices with a high content of fat and especially in the case of follow-up products such as peanut butter or pistachio paste the fat content and the consistency of these matrices are of great importance for the extraction procedure. After technological processing, these products consist of a lipid-phase with incorporated particles that are contaminated with aflatoxins. Aflatoxins are practically not fat soluble, thus a sufficient extraction is crucial to free any possible incorporated aflatoxins.

Chloroform (as a fat soluble solvent that also dissolves aflatoxins) was not considered to be used due to its toxicity and its incompatibility for further immunoaffinity clean-up procedures.

Aqueous acetone[1] was found to be a sufficiently fat soluble solvent to penetrate the fat phase of peanut or pistachio products and dissolve the aflatoxins. However it was not considered as an appropriate extractant (even though a rapid dissolution of the fat and the aflatoxins was observed), since the dilution of the filtered extracts with phosphate buffered saline or water [2] separated the dissolved fat and formed fine emulsions, which required additional clean-up steps and which did not occur with aqueous MeOH.

The extraction by high-speed blending with aqueous MeOH together with a non-polar solvent (hexane or cyclohexane) was found to be the most suitable extraction mode.

[1] Acetone-water [8+2]

[2] See Chapter 2.1.2

A subsequent filtration of the homogenized extract (emulsion of the non-polar solvent and the aqueous MeOH) allowed the successful separation of the non-polar solvent. The fat containing phase remained in the filter cake, while the aqueous filtrate (containing the extracted aflatoxins) was directly ready for further analysis. However, any delay in the filtration resulted in a separation of the emulsion and the formation of two layers in the filtrate.

The reliability of this extraction procedure was checked by the analysis of certified reference material using aqueous MeCN [3] and the modified MeOH extraction. As can be seen in Table 2.8 the determined values were in the certified range for both types of extractions, while both values obtained by triple determination did not differ significantly from the mean value according to a performed t-test.

Other alternatives such as sample de-fatting by Soxhlet [89] were not considered since they are time consuming and offer no analytical advantage.

Extraction of interfering matrix components Generally it is desired to extract the analyte specifically without other undesired matrix components, thus having preferably an analyte specific extraction.

Therefore the co-extraction of matrix components is another aspect in the development of an analytical methods. As discussed before, extractants based on aqueous acetone extract significantly less undesired matrix components from certain food matrices due to their less polar nature. This might be of interest concerning further clean up procedures, in which these components must be further separated from the analyte.

However, most analytes can be sufficiently purified by an immunoaffinity clean-up step. Thus, large amounts of co-extracted components as they occur in aqueous MeOH extracts are, with some rare exceptions, of minor relevance

[3] Aqueous MeCN has been widely used and discussed to be superior than aqueous MeOH for the extraction of aflatoxins in peanut butter

CRM-385	**MeOH extraction + hexane (n=3)**	**MeCN extraction (n=3)**
(7.0±0.8 μg/kg)	6.3 μg/kg	6.5 μg/kg

Table 2.8: Analytical results for certified reference material (peanut butter with a certified value of 7.0±0.8 μg/kg) obtained with different extractants

due to the highly specific affinity of the analyte to the antibody.

Animal feed containing citrus pulp Citrus pulp is a common constitutent in animal feed and has been described in literature to cause interferences with aflatoxins by determination with TLC and with HPLC [62, 90–92]. To a certain degree these interferences could be minimized in HPLC by derivatization with bromine rather than with iodine [90].

However chromatograms obtained from extracts of aqueous MeOH can still contain sufficient amounts of interfering citrus pulp even when purified by immunoaffinity. The reason for this interference can be assumed to be that citrus pulp might concentrate on the immunoaffinity gel and elutes together with the aflatoxin when washed out with neat MeOH.

Aqueous acetone has previously been shown to be superior in specific sample extraction for other matrices (Table 2.8) and has shown to sufficiently suppress interferences in chromatogram derived from citrus pulp containing extracts when further cleaned-up by an immunoaffinity step (Figure 2.10).

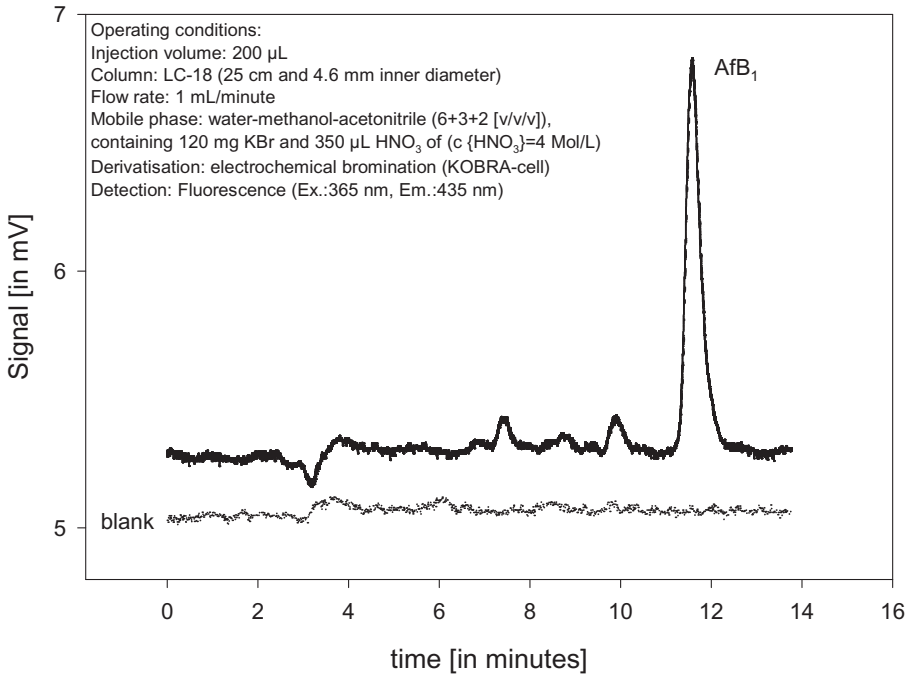


Figure 2.10: Chromatogram of an animal feedingstuff extract at approximately 1 μg/kg aflatoxin B_1

2.1.2 Sample Preparation

After extraction from the sample matrix, the aflatoxins have to be further isolated from any co-extracted matrix constituents. This is commonly done by solid phase extraction [93] or more recently by immunoaffinity clean-up procedures [61], while the latter procedures are the method of choice. Therefore the robustness and reliability of the immunoaffinity clean-up step for the here described matrices and methods were specifically evaluated. Generally the filtered extracts were diluted with PBS and directly applied on the IAC. In cases of considerable precipitation during the dilution process the extracts were filtered to avoid clogging of the IACs.

Sensitivity of the immunoaffinity column against organic solvents The nature of organic solvent is a critical parameter since the aflatoxin antibodies in the IAC are sensitive towards organic solvents. Several diluted extractants as well as immunoaffinity loading and elution procedures for mycotoxins have been reviewed [61]. Methanol concentrations of 5% up to 30% in the diluted extracts used for IA-clean up have been reported, while acetone concentration were found to be more critical for recovery, thus concentrations of 1% already might cause losses of aflatoxin G_2, while concentrations of >20% cause significant losses of aflatoxin B_2. It was found that final acetone concentrations of approximately 2% were suitable for most IACs and minimise undesired losses [78].

However, the development of IACs is an ongoing process and it was expected that the quality of IACs has changed over the time and even from product to product. Therefore the reviewed procedures were taken as a *guideline* for an in-house IAC testing.

The used IACs here were specified by the supplier not to be used with MeOH concentrations higher than 10%, while for diluted acetone solutions no reliable data was available [4]. Since it was intended to use acetone-water for the extraction of animal feed the robustness of the IACs was tested with mixed standard solution of aflatoxins and solutions of MeOH (10%) and various acetone concentration.

Aflatoxins in 10% MeOH were found to result in no significant loss in immunoaffinity performance (recovery), even if large volumes up to 100 mL were applied (Figure 2.11), thus indicating the suitable stability indicated by the supplier. Even solutions of 30 mL with a content of 15% MeOH have been shown to result in no relevant loss in performance (recovery of >90% for all aflatoxins), indicating a sufficient margin of robustness. Higher concentrations than this were not investigated in order to remain in the area of recommendation by the supplier.

[4]Rhone-Diagnostics *Easy Extract columns*

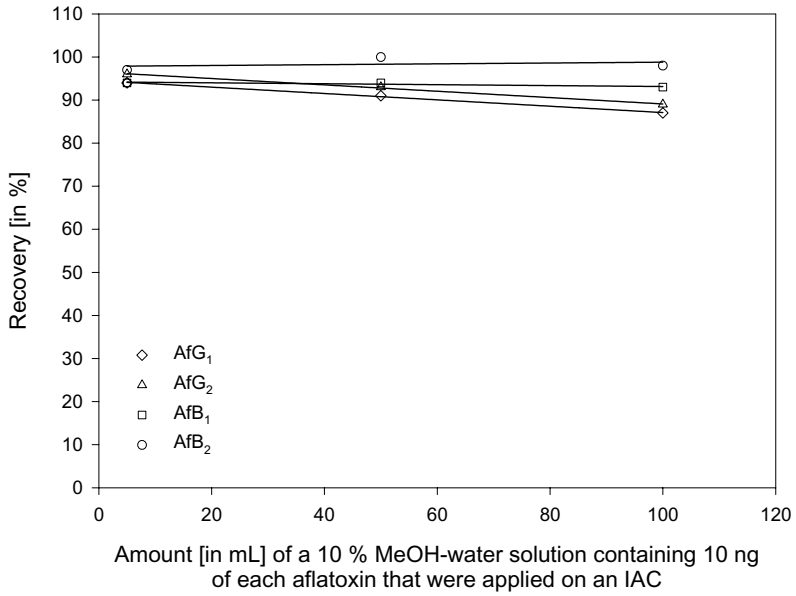


Figure 2.11: Robustness of IACs towards aqueous MeOH solutions

The application of diluted MeCN or acetone does not allow such concentrations as were used for MeOH or even in such large amounts of extract aliquots. MeCN was not tested for this purpose since it was previously excluded as an extractant, while acetone was directly compared with MeOH. As can be seen in Figures 2.12 and 2.13 the recovery for aflatoxins G_1 and G_2 decreases drastically at larger concentrations of acetone (>5%) or if larger volumes of solutions of aqueous acetone are applied. However the recovery for aflatoxin B_1 and aflatoxin B_2 remained fairly stable even at concentrations of more than 8%, thus indicating that for methods designed to determine aflatoxin B_1 only[5], acetone extractants were suitable up to a concentration of around 8% for the IAC-type used, with a sufficient margin of robustness even if volumes of 50 mL were applied on the IAC.

Washing procedures for the IAC Generally IACs are washed with water, PBS, or better with a solution that has the same composition as the diluted extract. The latter helps to avoid precipitation of diluted matrix

[5]A regulatory limit for cattle feed is foreseen to be established only for aflatoxin B_1

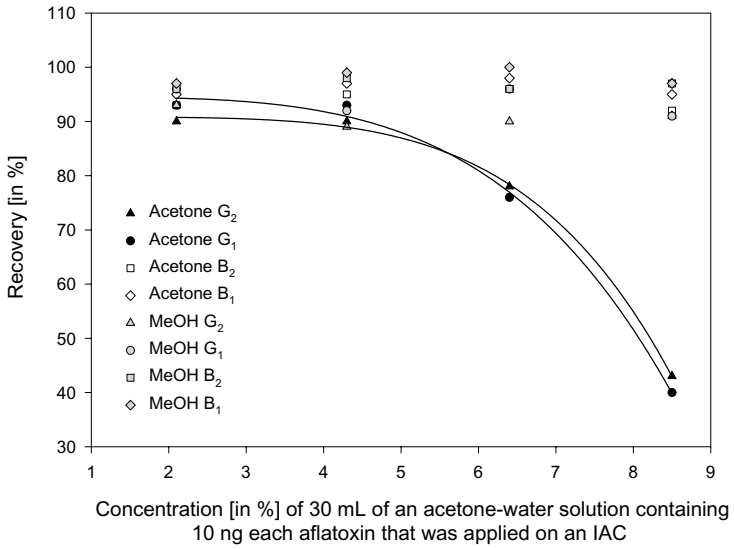


Figure 2.12: Sensitivity of IACs to organic solvents (depending on the concentration)

residues in the IAC gel. However washing procedures for further HPLC determination were found to be robust, since the aflatoxins get sufficiently separated from extract residues during the HPLC run in most of the cases. Therefore washing with 10 mL - 15 mL of water was sufficient for HPLC.

For further TLC separations the washing procedure was more critical and had to be modified. Not sufficiently purified aflatoxins (after IA-clean up) caused several problems: During evaporation of the IAC eluate precipitated extract residues were found to be difficult to re-dissolve in the spotting solution. In addition spotted residues inhibited the proper penetration of initial spots by the mobile phase, thus causing aflatoxin spot deformations (heart shaped spots) and interferences.

A sufficient purification was obtained when the IACs were first washed with the application solution containing 0.5% of Tween-20 (a detergent), followed by water to remove any detergent residue.

Elution of aflatoxins from the IAC The elution of the purified aflatoxins is generally performed with MeOH, MeCN or aqueous dimethyl sulfoxide [61]. Due to its toxicological data, its low boiling point and low elution power (compared to MeCN) MeOH was the preferred solvent in this study. The aflatoxins were eluted in a two-step procedure to allow the detachment of the aflatoxins from the antibodies in the first step (application of 0.5 mL on the IAC), while after 1 minute the dissolved aflatoxins were washed from the IAC completely with a second portion of approximately 1 mL of MeOH.

2.1.3 Determination by HPLC

Mobile Phase

Chromatographic systems for the determination of aflatoxins are influenced by several parameters. Even though HPLC-columns (type, brand or dimension), injection volume, mobile phase composition, flow-rate and temperature are often exactly specified in literature, it can be observed that differences can occur in chromatograms between laboratories or even within one labo-

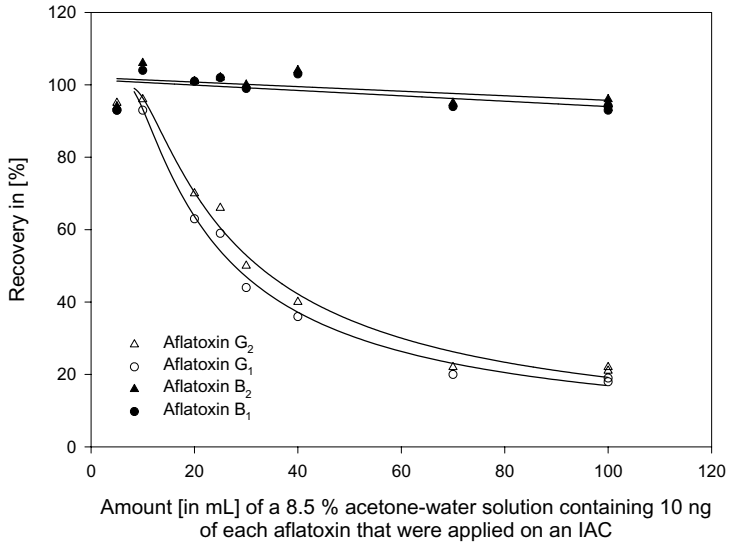


Figure 2.13: Sensitivity of IACs to organic solvents (depending on the volume applied)

ratory at different times.
Several mobile phases based on different ratios of water, MeOH and MeCN have been described in the literature for reversed-phase separations of aflatoxins (Table 2.9). Most authors did not indicate reasons for the selection of the mobile phase used or even chromatographic system, while can be assumed that systems were optimized for short retention times with full baseline separation of the peaks concerned.

Ref.	**Mobile phase composition and HPLC column type**				
	MeCN [Vol%]	H_2O	MeOH	MeCN	Column
[97]	33	670 [mL]	0 [mL]	330 [mL]	Phenyl
[85]	30	580 [mL]	120 [mL]	300 [mL]	ODS1
[101]	22	630 [mL]	150 [mL]	220 [mL]	RP-18
[169]	20	600 [mL]	200 [mL]	200 [mL]	LC-18
[90]	17	1300 [mL]	700 [mL]	400 [mL]	RP-18

Table 2.9: Composition of selected mobile phases with different MeCN content *The values for Vol% are not corrected for volume contraction of the final solution.*

The focus of the elaborated mobile phase development was orientated on published data and considered to obtain sharp peaks with a resolution of $\geq$1.25[6] (to limit errors during integration [94]), favorable capacity factors (k') of 1 - 5 [95], while reducing the amount of MeCN in the mobile phase. Low amounts of MeCN were favorable, since MeCN is a toxic solvent and the membrane of the post derivatization system (KOBRA[7]-cell) is known to be sensitive towards higher concentrations of MeCN.

Mobile phases on the base of water and MeOH were tested initially. However it was found that these binary systems did not allow the separation of the aflatoxins in a reasonable time of analysis. The amounts of MeOH needed to allow the separations in an acceptable amount of time, lead to the merging of aflatoxin peaks, due to the relative low plate numbers that were obtained with these binary systems. Mobile phases out of water and MeCN offered sharper peaks for all aflatoxins, however the peaks could not be separated

[6] According to the technical annex of the project proposal the maximum height of the valley of 2 overlapping peaks had to be less than 10% of the peak height of the smaller peak.

[7] Kok's bromination apparatus

fully due to similar retention times in these mobile phases. A mobile phase composed of water, MeOH and MeCN (600 + 300 + 200 [v/v/v]), as it was previously described [81], has been found to offer a sufficient separation with a least amount of MeCN for the in-house HPLC system. This mobile phase offered the desired peak separation within an analysis time of 15 minutes with a relatively small amount of MeCN of less than 20 vol%.

Injection Volume and Injection Solvent

The HPLC injection volume can be a critical parameter that can highly influence the performance of an HPLC system. On one hand a large injection increases the fraction of the analyte, while on the other hand large injection volumes imply that the starting zone at the beginning of the chromatogram can lead to losses of performance.

Paste of peanuts, pistachios, figs, and paprika powder The current regulatory limits for these food items are 2 μg/kg for aflatoxin B_1 and 4 μg/kg for total aflatoxins [46]. A loop volume of 200 μL allows, in combination with the above described sample preparation, the injection of a sample equivalent of 0.2 g (200 pg aflatoxin per injection at half of the regulatory limit). This amount results in sufficiently large peaks, while at a flow-rate of 1 mL per minute of mobile phase the starting zone (injection zone) is equivalent to 0.2 min peak width prior separation. A full baseline separation of all aflatoxin peaks was obtained when purified extracts from naturally contaminated sample material were analysed (Figures 2.14 to 2.17).

Infant formula The regulatory limit for infant formula is foreseen to be 0.1 μg/kg aflatoxin B_1. This would result in much a smaller fraction of 1/20 for the injection according to the above selected parameters. Such a small amount was found not to be sufficient for a broad application of a method, as desired in a collaborative trial.

As known from literature [94] an important contribution for errors at measurements close to the LOD or LOQ is improper integration of signals due to baseline noise.

Officially the limit of detection is defined as: »*...the smallest measured content from which it is possible to deduce the presence of the analyte with a reasonable statistical certainty.*«, while the limit of quantification is defined as: »*...the smallest measured content above which a determination of the analyte is possible with a specified degree of accuracy and repeatability (within laboratory).*« [96].

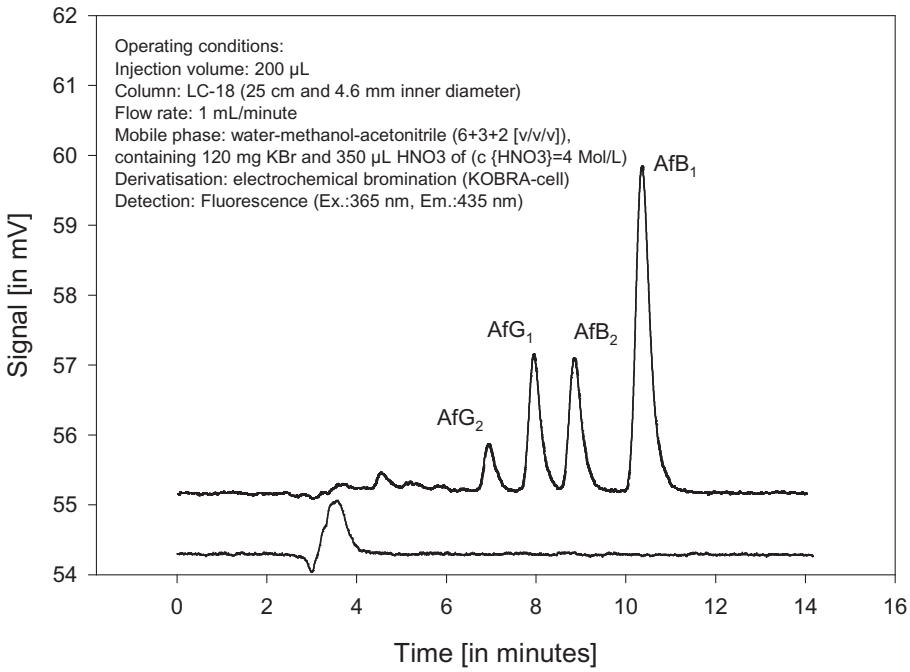


Figure 2.14: Chromatogram of a peanut butter extract at approximately 1 μg/kg aflatoxin B_1

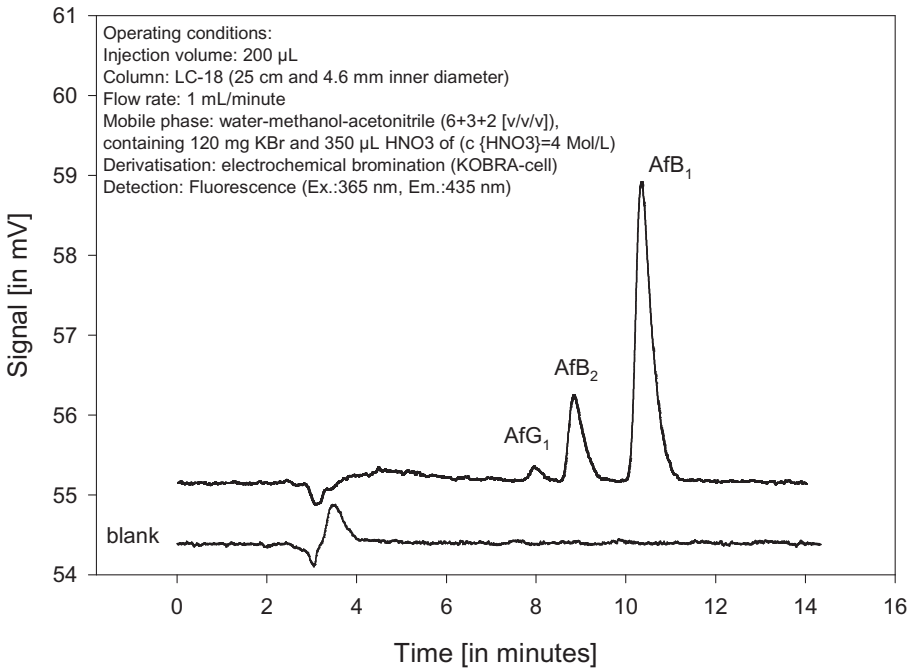


Figure 2.15: Chromatogram of a pistachio paste extract at approximately 1 μg/kg aflatoxin B_1

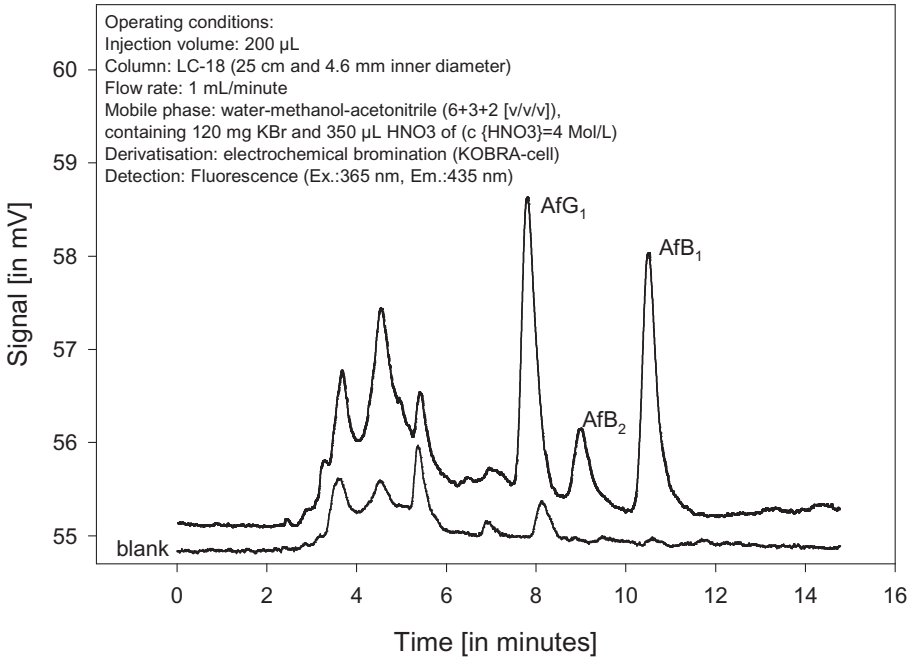


Figure 2.16: Chromatogram of a paprika powder extract at approximately 1 μg/kg aflatoxin B_1

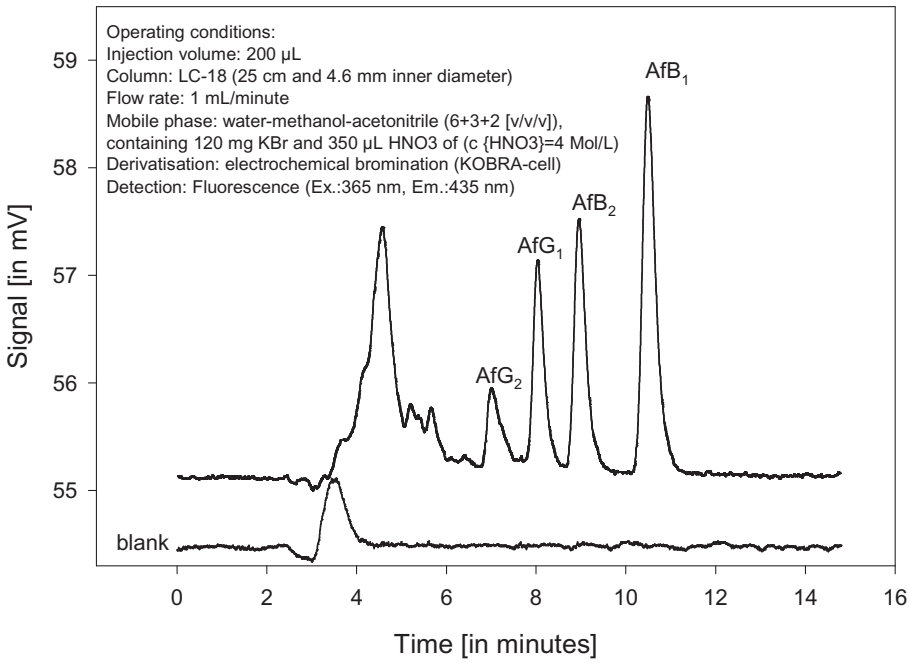


Figure 2.17: Chromatogram of a fig paste extract at approximately 1 μg/kg aflatoxin B_1

Therefore, an experiment was carried out to find out the performance characteristics of the chromatographic and the detection system. Therefore multiple injections of several aflatoxin B_1 solutions were made close to the assumed LOQ. The RSD of these signals for each level was then plotted against the corresponding amount. As can be seen in Figure 2.18 the amount that was needed for sufficiently precise measurements is about 20 pg aflatoxin per injection. However to obtain a certain margin of safety, an amount of approximately two to three times the measured value was targeted.

A simple strategy to assure the detection of sufficiently large amounts of aflatoxin was the injection of larger volumes then 200 μL, as was previously done. Such a procedure will allow to increase the amount of aflatoxin B_1 for the injection, without work intensive procedures such as evaporation, which is known to be an additional source of errors [97] and also more difficult to automate.

In a calculation model the amount of AfB_1 that can be injected by simply increasing the injection volume to 1000 μL was determined:

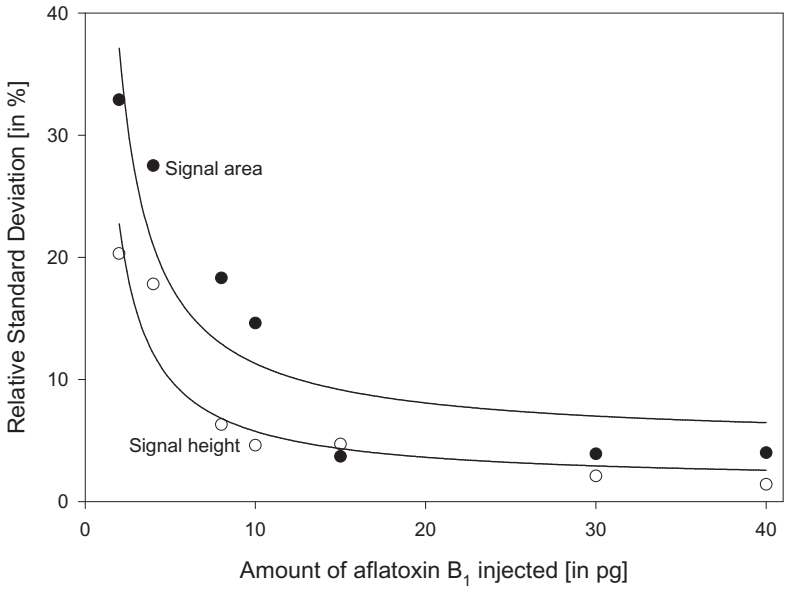


Figure 2.18: Signal precision and aflatoxin B_1 amount for the in-house HPLC-fluorimeter (detector) system

$$\mathrm{AfB_1} = \frac{\text{weight [g] * aliquot for IAC [mL] * inj. vol. [mL] * contamin.}[\mu\text{g/kg}]}{\text{extractant [mL] * elution after IAC [mL]}}$$

$$\mathrm{AfB_1} = \frac{50\text{g} * 10\text{mL} * 1\text{mL} * 0.1\mu\text{g/kg}}{200\text{mL} * 5\text{mL}}$$

$$\mathrm{AfB_1} = 0.05\text{ng} = \mathbf{50\ pg}$$

As can be seen, an absolute amount of 50 pg of aflatoxin B_1 can be injected this way. However when large volumes are injected into a HPLC system, the performance of the separation is likely to suffer due to peak broadening. A common procedure to overcome this is the injection of the analyte in a solvent sufficiently lower in elution power than the mobile phase. Thus the aflatoxin B_1 and other substances will concentrate during injection in the first layers of the HPLC column, which results in a better HPLC separation thereafter.

It was reported that the composition of the injection solvent and of the mobile phase were identified to be crucial in terms of separation efficiency [98]. This holds especially true for large injection volumes [99]. Therefore the interaction of the mobile phase and the injection solvent was evaluated in terms of plate numbers (N) and baseline separation of aflatoxin B_1 with other substances. It could be demonstrated that the desired volume of 1000 μL can be injected without any significant loss of performance, provided that the compositions of the mobile phase and the injection solvent were chosen carefully. Figure 2.19 shows the resulting chromatograms for different water-MeOH solvents, while Figures 2.20 and 2.21 reflect the relationship of the signal yield and the platenumber for the corresponding chromatograms.

It was concluded that a water content of 65 vol% of water or even higher (1.75 mL MeOH form the elution of the aflatoxin + 3.25 mL water) in the injection solution was sufficient to allow a sufficient separation.

Derivatization and Detection

Aflatoxins are highly fluorescent compounds, while the flourescence properties of the single aflatoxins differ in several aspects. Besides the the slightly different emission maxima of each aflatoxin one of the most important characteristics is the solvent depending fluorescence quenching of some aflatoxins. AfB_1 and AfB_2 are known to undergo quenching of fluorescence in chlorinated solutions, while AfB_1 and AfG_1 undergo quenching when present in aqueous

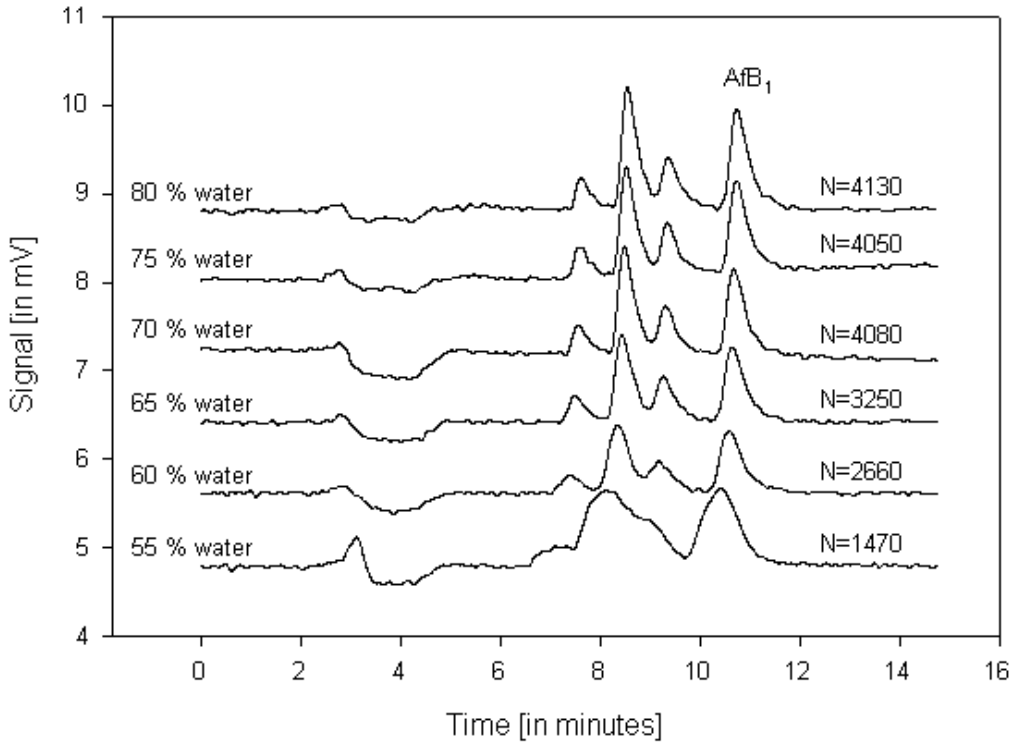


Figure 2.19: Influence of the injection solution on chromatograms

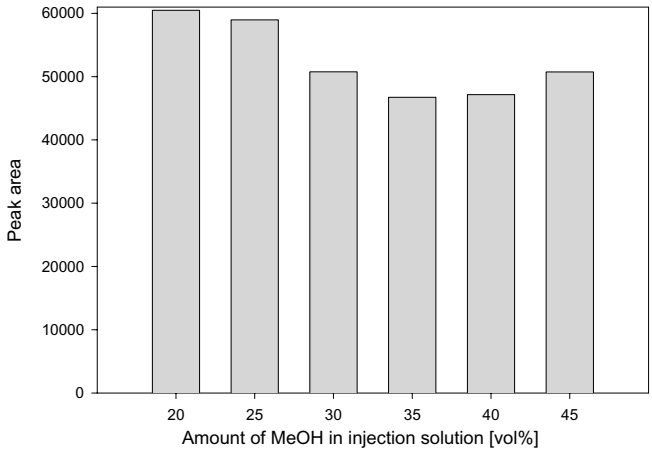


Figure 2.20: Influence of injection solution on signal area

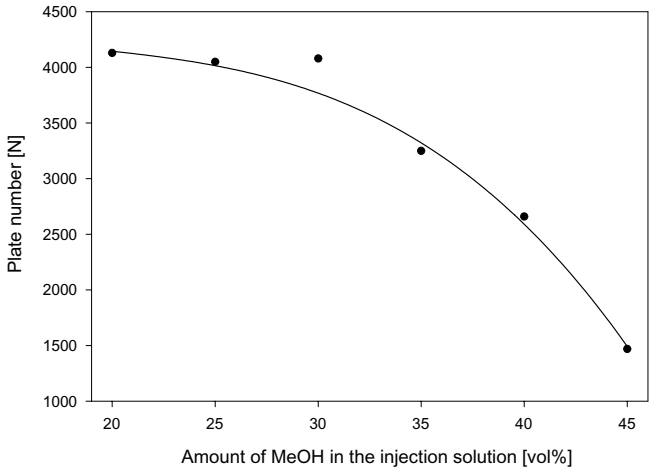


Figure 2.21: Influence of injection solution on plate numbers

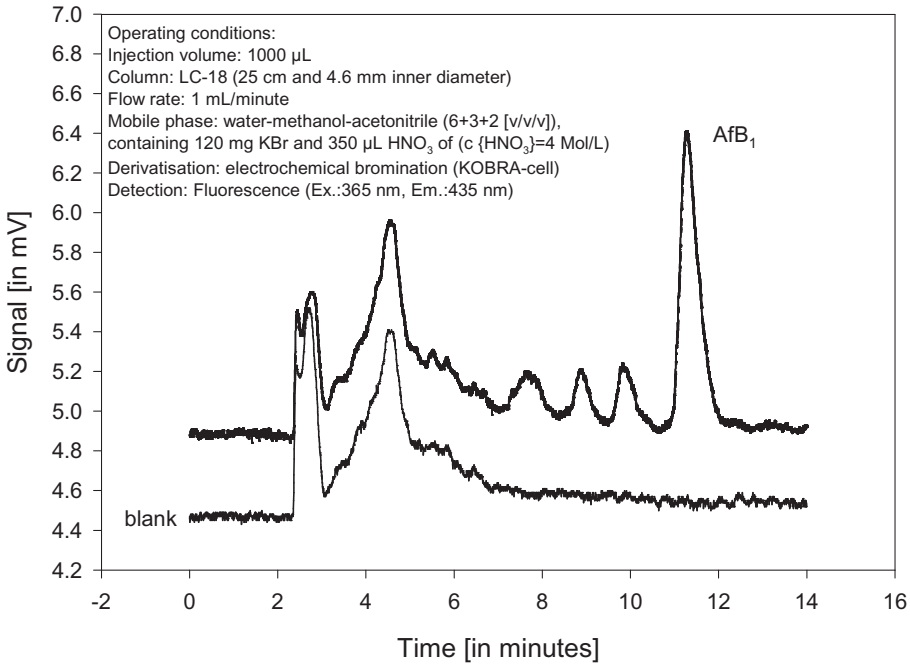


Figure 2.22: Chromatogram of infant formula
at a contamination level of approximately 0.1 μg/kg aflatoxin AfB_1

solvents [1]. Nowadays only the latter case is of importance, since modern chromatography of aflatoxins is based on RP-HPLC with aqueous mobile phases.

Several methods have been proposed to suppress the described quenching in aqueous mobile phases. The main principle is the saturation of the furanic ring. This can be performed by pre-column derivatization of AfB_1 and AfG_1 to their hemi-acetals AfB_{2a} and AfG_{2a} with trifluoro-acetic acid (TFA) (Figure 2.23) [100]. However this derivatization type exhibits several disadvantages [1, 62] and is nowadays replaced by post column derivatization (PCD) procedures, which are superior to pre-column derivatization.

A PCD method for the formation of the fluorescent hemiacetals is UV-radiation of the aflatoxins in a transparent coil [101].

Other commonly used PCD procedures are based on the reaction of the aflatoxins with halogenides such as iodine[8] or bromine[9] [62]. Figures 2.24 and 2.25 illustrate the resulting reaction.

Another PCD method that lately found some interest is the post column addition of (α-, β-, or heptakis-2,6-*o*-dimethyl-) cyclodextrine to the mobile phase [102]. However, this method was not further evaluated, since it seemed to offer no advantages compared to post column bromination or iodination. Long term costs (routine analysis) for the cyclodextrines were found to be higher than for bromination agents, while the method has never been re-

[8]In form of a saturated aqueous iodine solution.

[9]In form of electrochemically derived bromine (KOBRA-cell ®) or a bromination agent (solution of *Pyridinium Bromide Perbromide* PBPB), Sydenham96

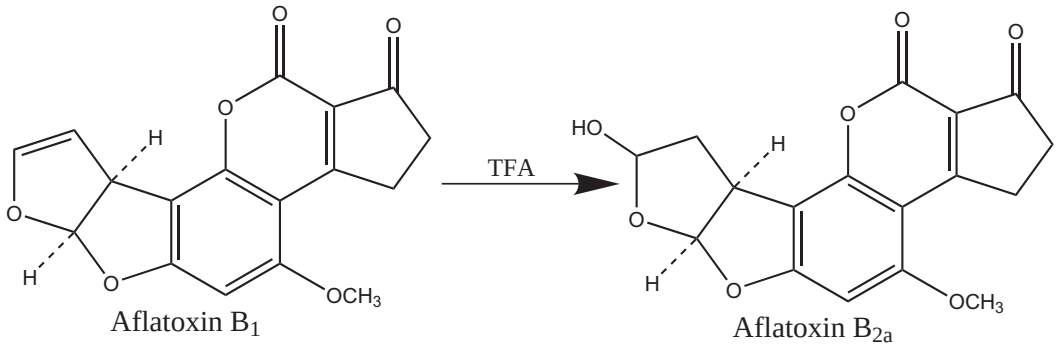


Figure 2.23: Conversion of aflatoxin B_1 to aflatoxin B_{2a}

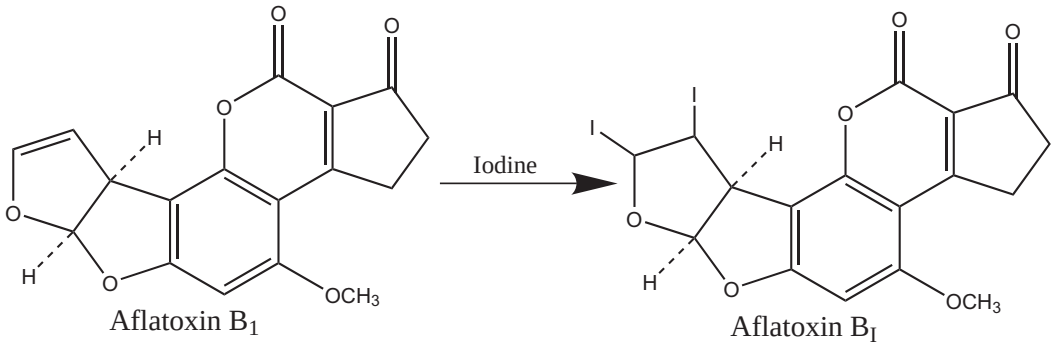


Figure 2.24: The iodine derivative of aflatoxin B_1

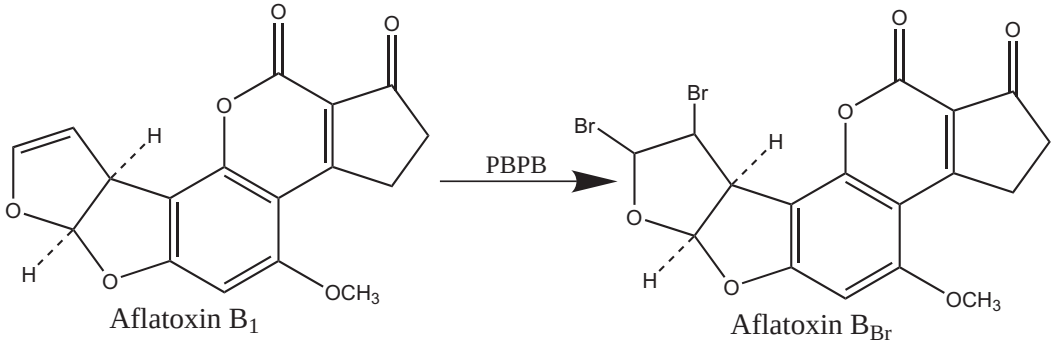


Figure 2.25: The bromine derivative of aflatoxin B_1

ported to be used for routine analysis.

From the methods discussed only bromination, iodination, and UV-light were considered as possible candidates, since they have been applied for routine work. However, bromination can be achieved by two different approaches. One method is the addition of a bromination agent (*Pyridinium Bromide Perbromide* PBPB) to the mobile phase, while the second method is the production of electrochemically generated bromine. This is achieved by addition of Potassium bromide to the mobile phase, which releases bromine in a special generator cell. A schematic draw of the KOBRA-cell ® is given in Figure 2.25.

The conditions and requirements of the two bromination and the iodination PCD are listed in Table 2.10. As can be seen, post column bromination is superior in terms of the equipment required and in reaction time, while iodination requires harsher reaction conditions (temperature and time).

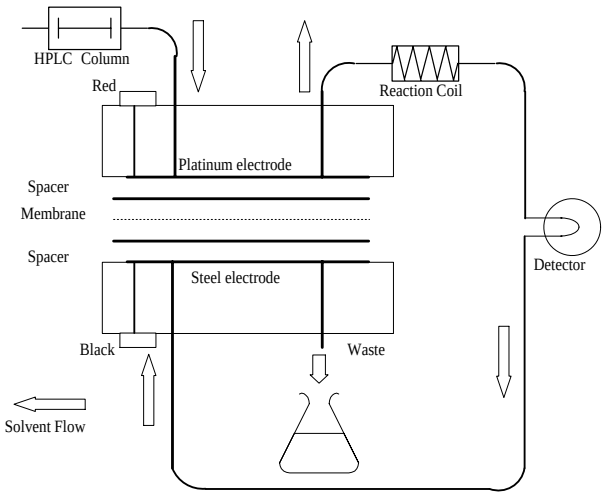


Figure 2.26: Schematic layout of the KOBRA-cell ®

Parameter	Iodine	PBPB	Electrochem.
Flow rate	1 mL + 0.6 mL reactant	1 mL + 0.3 mL reactant	1 mL with no reactant
Reaction solvent	Saturated iodine in water	50 mg PBPB in 1L water	KBr and HNO_3 added to mobile phase
Stability	Approximately 1 day	Up to 4 days (in the dark)	unlimited
Reaction coil	6 meters	50 cm	50 cm
Reaction temperature	70 degrees Celsius	Ambient temperature	Ambient temperature
Extra devices	Oven or post column reactor	none	Cell with diaphragma
Signal yield	Smaller than PBPB	Bigger than iodine	Bigger than PBPB and iodine
Known disturbances	iodine crystallisation	degradation of PBPB if stored improperly	none

Table 2.10: Comparison of post column procedures based on iodine and bromine

Furthermore the UV-derivatization was compared with post column bromination (KOBRA-cell ®). It was found that the time for a chromatographic run is about 2.5 minutes longer (approximately 15%) for the UV-derivatization (Figure 2.27). This is due to the larger void volume of the radiation coil compared to the reaction tube needed for bromination. Since UV-derivatization offered no advantages compared to bromination in terms of fluorescence amplification, post-column derivatization (PCD) by bromination was finally considered as the method of choice.

Since the generation of electrochemically derived bromine required a special device (KOBRA-cell ®)[10], the two bromination approaches were compared. As can be seen in Table 2.11 the precision data and the responses of both methods are comparable and satisfactory for both methods. Therefore it was foreseen to consider both bromination methods for the collaborative trial.

[10]Currently only available from one manufacturer

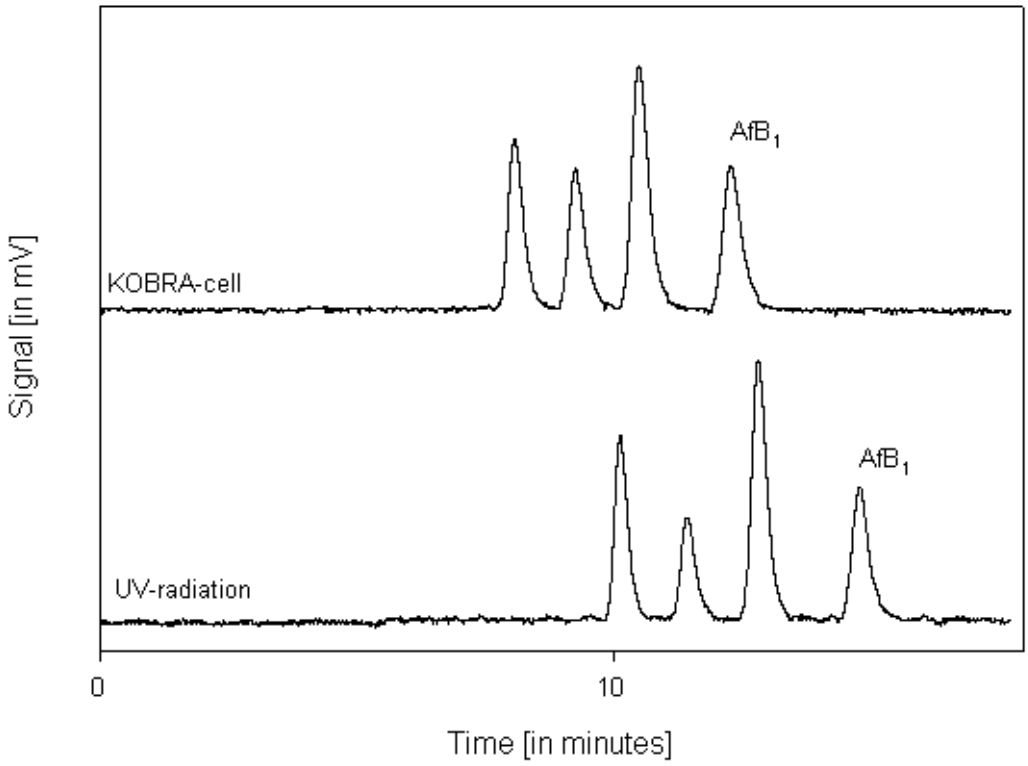


Figure 2.27: Chromatograms of aflatoxins with PCD
by bromination or UV-ratidation

Aflatoxin	**KOBRA** (Response)	**PBPB** (Response)	**KOBRA** (RSD of signal)	**PBPB** (RSD of signal)
B_1	1	0.90	1.2	2.8
B_2	1	0.85	0.6	1.4
G_1	1	0.68	0.6	1.9
G_2	1	0.73	0.3	3.8

Table 2.11: Comparison of electrochemical(KOBRA)
and PBPB derivatization

In-house Performance Data for the HPLC Methods

In-house validation was carried out to verify the feasibility of the method design. The selected parameters for in-house validation were mainly taken from the criteria that are laid down by the European Standardization Committee (CEN) [51] and that were mentioned in the technical annex of the project [69].

These figures included data for the LOD, LOQ, recovery (target level), precision (»repeatability« [n=10]) for naturally contaminated material and fortified material. Figures had to be established for all matrices and aflatoxins mentioned at the target level. As can be seen in Tables 2.12 to 2.16 - for various food items - and in Tables 2.17 to 2.18 - for infant formula - the in-house performance data obtained was satisfactory and gave strong indication that the methods are suitable for further testing in collaborative trials.

Parameter:	**AfG_2**	**AfG_1**	**AfB_2**	**AfB_1**	**AfB_1 target values**
LOD [in μg/kg]	0.05	0.05	0.12	0.09	0.5
LOQ [in μg/kg]	0.07	0.07	0.18	0.13	1.0
Repeatability [in %]	-	8	9	8	15
Precision (nc) [in %]	3	2	5	4	15
Precision (spike) [in %]	3	3	4	5	15
Recovery (0.5x) [in %]	85	82	80	88	70-120
Recovery (1.0x) [in %]	100	90	87	112	70-120
Recovery (1.5x) [in %]	95	93	86	86	70-120

Table 2.12: In-house performance characteristics for aflatoxins in figs

Parameter:	**AfG_2**	**AfG_1**	**AfB_2**	**AfB_1**	**AfB_1 target values**
LOD [in μg/kg]	0.06	0.05	0.05	0.05	0.5
LOQ [in μg/kg]	0.09	0.08	0.08	0.08	1.0
Repeatability [in %]	-	-	-	12	15
Precision (nc) [in %]	-	4	9	6	15
Precision (spike) [in %]	4	6	9	14	15%
Recovery (0.5x) [in %]	99	74	77	101	70-120
Recovery (1.0x) [in %]	80	72	71	75	70-120
Recovery (1.5x) [in %]	86	74	80	107	70-120

Table 2.13: In-house performance characteristics for aflatoxins in paprika

Parameter:	**AfG_2**	**AfG_1**	**AfB_2**	**AfB_1**	**AfB_1 target values**
LOD [in μg/kg]	0.05	0.03	0.03	0.03	0.5
LOQ [in μg/kg]	0.07	0.05	0.04	0.04	1.0
Repeatability [in %]	-	13	-	11	15
Precision (nc) [in %]	-	-	-	13	15
Precision (spike) [in %]	3	4	4	5	15
Recovery (0.5x) [in %]	86	86	70	70	70-120
Recovery (1.0x) [in %]	100	98	85	86	70-120
Recovery (1.5x) [in %]	117	115	93	91	70-120

Table 2.14: In-house performance characteristics for aflatoxins in pistachios

Parameter:	**AfG_2**	**AfG_1**	**AfB_2**	**AfB_1**	**AfB_1 target values**
LOD [in μg/kg]	0.05	0.03	0.03	0.03	0.5
LOQ [in μg/kg]	0.08	0.05	0.05	0.04	1.0
Repeatability [in %]	-	-	-	14	15
Precision (nc) [in %]	-	-	-	8	15
Precision (spike) [in %]	1	1	2	5	15
Recovery (0.5x) [in %]	79	74	87	75	70-120
Recovery (1.0x) [in %]	85	85	79	82	70-120
Recovery (1.5x) [in %]	87	80	90	82	70-120

Table 2.15: In-house performance characteristics for aflatoxins in peanut butter

Quotient %		**Quotient %**		**Quotient %**		**Quotient %**		**Level**
$q = \frac{area}{conc.}$ AfG_2		$q = \frac{area}{conc.}$ AfG_1		$q = \frac{area}{conc.}$ AfB_2		$q = \frac{area}{conc.}$ AfB_1		in μg/kg
323704	**105**	276873	**110**	565850	**103**	292779	**105**	1
310129	**100**	257696	**102**	565350	**103**	284342	**102**	1.5
314423	**102**	255343	**101**	555844	**101**	276738	**100**	2
308112	**100**	248923	**99**	546738	**100**	278251	**100**	2.5
305925	**99**	244383	**97**	541465	**99**	273937	**98**	3
302712	**98**	240649	**95**	534242	**97**	271485	**98**	3.5
301415	**97**	242566	**96**	537824	**98**	270270	**97**	4
mean		mean		mean		mean		
309489		252348		549616		278257		

Table 2.16: In-house performance characteristics for aflatoxins - calibration curve

Required parameter	**Performance characteristics for AfB_1**	
	required	**found**
Precision (nc) [in %]	15	14.8
Precision (spike) [in %]	15	11.6
Repeatability [in %]	45	12.5
LOD [in μg/kg]	0.05	0.02
LOQ [in μg/kg]	0.10	0.04
Calibration variation [in %]	90-110	91-106
Recovery - 0.5x [in %]	50-120	61-66
Recovery - 1.0x [in %]	50-120	50-71
Recovery - 1.5x [in %]	50-120	63-67

Table 2.17: In-house performance for infant formula
In-house performance characteristics for aflatoxin B_1 in infant formula

Level [μg/kg]	$q = \frac{area}{conc.}$	**%**
0.05	1191	91
0.15	1303	99
0.25	1386	106
0.35	1382	105
0.45	1290	98
0.05 - 0.45	Mean: 1310	Range: **91-106**

Table 2.18: In-house performance characteristics for AfB_1 - calibration curve

2.2 Determination by TLC

2.2.1 Development of a TLC Method

Thin-layer chromatography (TLC) is still underappreciated and the method of choice in cases where HPLC is not available and the precise determination of aflatoxins are required [19, 93, 103–106]. Developing countries are important exporters of food and food products that are subject to aflatoxin contamination (e.g. figs, spices, pistachios and peanuts) [20, 21], thus a simple and precise method such as TLC is a crucial tool for quality control prior to export. Most of the currently available (and all official) TLC methods still require chlorinated solvents such as dichloromethane or chloroform in the mobile phase, as extraction solvent or for sample clean up [59, 107, 108].

However chlorinated solvents are considered to be ecological hazards [109]; they are constantly being banned in laboratory routine work where possible.

Thus, a state-of-the-art TLC method, which is user friendly, easy to perform and renounces the use of chlorinated or other toxic solvents would be highly desirable for analysts who rely on TLC.

One approach considered the implementation of immunoaffinity columns, which are nowadays easily available in all parts of the world at a fair price. Therefore the implementation of the immunoaffinity clean-up step that has been applied for the above described HPLC method [83] was slightly modified for later TLC separation.

The combination of IAC and TLC has so far only been considered for the determination of aflatoxins at contamination levels of 10 μg/kg - 50 μg/kg [110] or more recently for the quantification of aflatoxin M_1 in milk[11], while the application for the variety of food items and contamination level described here has not been applied yet.

Sample Extraction

As described in Chapter 2.1.3, it is possible to quantify aflatoxin amounts of a few pg (absolute) by HPLC, however the fluorimetric determination of aflatoxins on TLC-plates requires significantly larger amounts for precise quantification.

As shown in chapter 2.1.2 aqueous MeOH is the only extractant that allows the application of larger fractions of sample extract on the IAC. Therefore the extraction procedure was not subject to modification for the TLC employed here.

Sample Preparation

Removal of interfering matrix traces Paprika powder samples which were analysed by TLC and purified with the described IA clean-up procedure for HPLC (Chapter 2.1.2) resulted in interferences on the TLC plate. It was found that a rapid and simple IA clean-up procedure, as used for HPLC, was not applicable for TLC determination. In addition, it was found that the evaporated IAC eluates from pistachios, peanuts and corn caused problems during re-dissolving and in spot symmetry after TLC development.

The reason for these interferences, which did not occur in HPLC, were due to the different separation mechanism of both systems (**normal-phase** TLC and **reversed-phase** HPLC).

[11] Personal communication with Sylviane Dragacci, AFSSA, France

The nature of these interferences was not further investigated, however, due to their colour of interfering substances, there was strong evidence that it was due to part of the natural and specific pigmentation (colour) of the sample material.

As already described in chapter 2.1.1, commonly used MeOH-water extractants are likely to extract more interfering matrix components than those based on acetone or MeCN, while the main advantage of MeOH-water is the application of large sample fractions for the IAC clean-up. Therefore the substitution of MeOH-water extractants was not considered, unless additional strategies would be required to assure a sufficient purification.

The following additional purification procedures were evaluated:

- Prior to IAC application, the diluted sample extracts were re-filtered with glass micro-fibre filter or with a combination of a glass micro-fibre and a Nylon ® filter, until the filtrate was clear. This filtration was identified as an important procedure for most sample materials (e.g. paprika powder, pistachio paste, peanuts and corn) to remove any traces of precipitate that occurred during the dilution of the extract.

- Another source for impurities was found when the practically purified aflatoxins were washed with water, as has been done without any problem for HPLC. Due to the different solubility, impurity traces remained in the IAC-gel. It was found that could be overcome with the following two-step washing procedure:

  - Washing with the diluted extractant[12] that contained 0.5% Tween-20® followed by
  - Washing with laboratory grade water.

Tween-20® is a commonly used non-ionic detergent[13], and has been shown to successfully purify IA-columns that were used for the clean-up of corn samples for subsequent fluorimetric measurement [111, 112].

The described procedure was tested for all matrices under discussion, and it was shown that all relevant interferences from paprika powder, pistachio paste, peanut butter, corn and fig paste were successfully removed (Figure 2.28).

[12]Solution with the same composition as the used for sample application on the IAC
[13]mono-sorbitan laureat

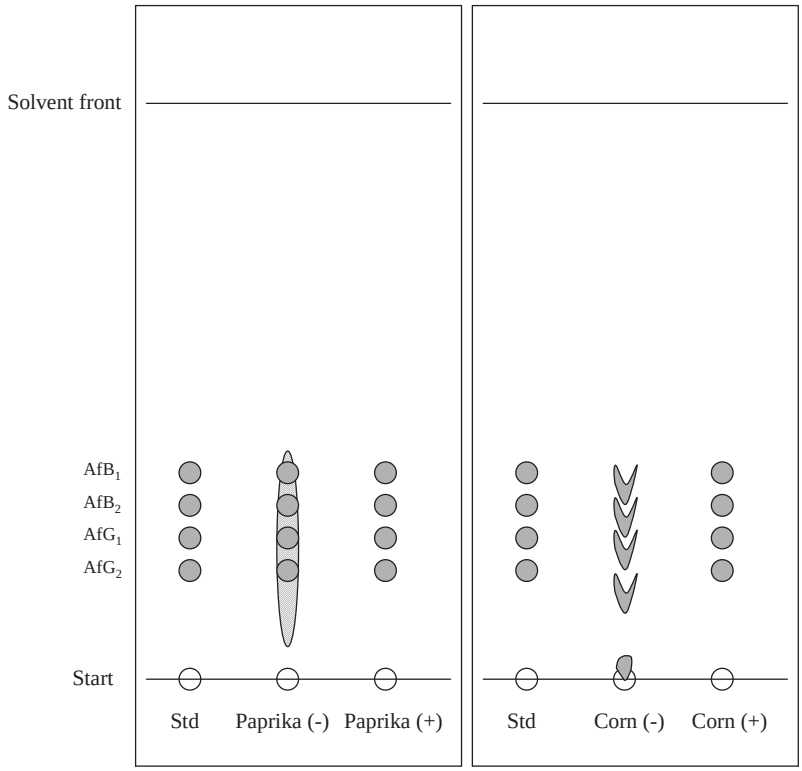


Figure 2.28: Thin-layer chromatograms of paprika powder and corn with (+) and without (-) the Tween-20® clean-up

Elaboration of a suitable spotting solvent It has been observed that MeOH is inappropriate for spotting of aflatoxins on silicagel TLC-plates. Due to its high elution strength, the aflatoxins would concentrate on the outer rim of the solvent front, which leads to unsuitable spots (Figure 2.30). Thus, an additional evaporation step was required for TLC separation. This allows to further concentrate the purified aflatoxins, while transferring them to a suitable solvent.

Loss of aflatoxins during evaporation Several factors that can lead to aflatoxin degradation during chemical analysis have already been identified in the past [64, 113, 114].

It was found that the pattern of aflatoxin losses can give information on the general principle of the observed loss. A degradation due to an alkaline environment will result mainly in a loss of AfG_1 and AfG_2, while UV-light induced degradation has shown to result in lower values for AfB_1 and AfG_1 mainly [64]. The reason for the latter case is the formation of hemiacetals (similar to the derivatization by TFA) as described in chapter 2.1.3, which only AfB_1 and AfG_1 can form. Alkaline degradation is due to the cleavage of the lactone, to which AfG_1 and AfG_2 are more susceptible, since they contain two lactone systems (Figure 2.29).

Even though only acid washed glassware was used for all procedures[14], it was found that recoveries from *fortified samples* yielded in unacceptable figures of 50% to 55% for aflatoxins AfG_1 and AfG_2, while recoveries for aflatoxins AfB_1 and AfB_2 were in an acceptable range of 80% to 85%.

On one hand recoveries and peak pattern obtained from evaporated methanol standard solutions revealed that no loss of aflatoxins occurred during the evaporation of aflatoxin standards in neat methanol. On the other hand, most of the other procedures that have been adopted from an HPLC method [83] have shown to be robust.

Therefore the degradation was thought to be due to other causes linked to evaporation, such as water or PBS residues in the IAC eluate (which are not present in neat MeOH standard solutions), or even traces of the Tween-20 or other unknown *washed-off* materials from the IAC.

For further investigation, aflatoxins in methanol were evaporated after addition of aqueous solutions that contained PBS and/or Tween-20®.

These experiments revealed that in most evaporated solutions an aflatoxin loss occurred, while the loss in neat MeOH was only marginal. However this

[14] All glassware that was used in this study was sufficiently acid washed with 10% sulphuric acid over night to remove any traces of alkaline substances and subsequently thoroughly washed with water.

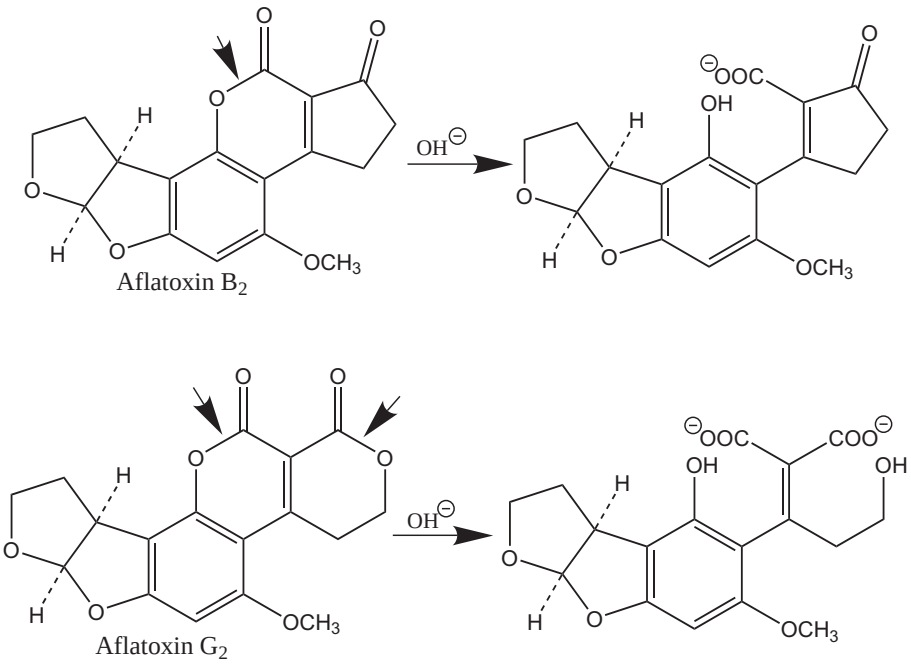


Figure 2.29: Degradation of aflatoxins in an alkaline environment

degradation could sufficiently be subdued with small amounts of formic acid during evaporation. A solution of formic acid - water - methanol (5 + 5 + 90 [v/v/v]) was found to be suitable, when 50 μL were delivered into the glass vials prior to transfer of the aflatoxin solution. The mentioned composition was chosen, since small amounts of formic acid could easily be passed into the vial without handling concentrated acids, while the water content inhibited the formation of methyl formiate.

Re-dissolving the aflatoxins Several TLC spotting solvents have previously been investigated with the aim to minimize spot size of the applied aflatoxins [115, 116]. However, several solvent mixtures were evaluated on the base of non-chlorinated solvents and solvents with a low toxicity. Important analytical criteria for the selection were the following:

- Sufficient solubility of the aflatoxins (to obtain an adequate recovery of the dried aflatoxins) [117],
- low boiling point (to achieve a fast evaporation of the solvent on the TLC-plate) and

- low elution strength (to avoid migration of the aflatoxins to the solvent front during application) [117].

One of the first candidate solutions chosen was a mixture of toluene-MeCN since aflatoxins are commonly stored in solutions of 95% to 98 % toluene-MeCN. However, next to the relative toxicity of MeCN, the boiling points of toluene (111°C) and of MeCN (82°C) are relatively high. Therefore solvent mixtures based on n-hexane (68°C) or cyclohexane (81°C) were evaluated as spotting solvents, while the boiling point of n-pentane (36°C) was considered to be too low for precise measurements (especially in warmer regions).

It was found that the presence of a sufficiently polar solvent, such as acetone (56°C) or MeOH (65°C) was essential to retrieve the dried aflatoxins in solution. Experimental data from evaporated aflatoxin standards, which were re-dissolved in various solvent candidates, showed that even an acetone concentration of 15% in n-hexane or cyclohexane only resulted in recoveries of 85% - 90% of the evaporated amount. Contrary to acetone, MeOH alone is not sufficiently miscible with n-hexane or cyclohexane, thus no relevant recovery data was obtained.

Experiments based on the addition of acetone (as modifier) and MeOH resulted in miscible solutions and offered excellent recoveries. Consequently, a solvent mixture of hexane-acetone-MeOH (90 + 5 + 5 [v/v/v]) was found to be most suitable in terms of recovery (98% - 101%) for all aflatoxins. Spot diameters were found to be between 2.0 and 2.5 mm when volumes of 100 μL were applied within 2 min (Figure 2.30). In addition, the mixture consists of solvents with relatively low toxicity compared to previously described spotting solvents [20].

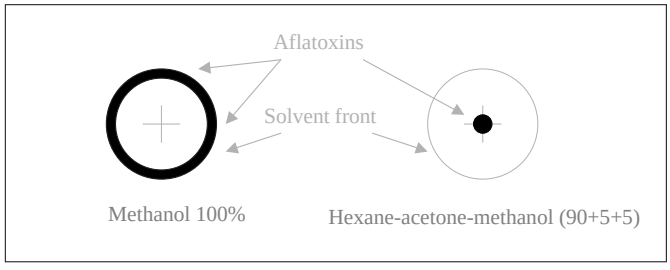


Figure 2.30: Evaluation of the elaborated spotting solvent and comparison with methanol

Due to the fairly low boiling point of the spotting solvent mixture, unwanted evaporation during the re-dissolving process had to be minimized. This was achieved by injecting the solvent into already sealed glass vials (containing the evaporated aflatoxins) with a micro-litre syringe. A volume of 150 μL had been selected, assuring that aflatoxins dissolve fully and only a neglectable amount of solvent would evaporate in the head space of the vial.

Application of the aflatoxins on the TLC-plate In a previous study different spotting procedures for aflatoxins were elaborated and it was reported that no significant difference in *precision* was found when solutions were sprayed or spotted [115].

However, while elaborating several spotting solvents and techniques, it was found that peak heights of classically applied aflatoxin spots (syringe or capillary) were approximately 15% to 30% smaller than those obtained by spraying procedures. It is remarkable that this effect was found for all spotting solution compositions. Hence it can be concluded that the way solvent is applied on the TLC-plate is an important factor for the fluorescence signal yield (Table 2.19).

Spotting solvent	**Classical spotting**				**Linomat (spraying)**			
	A	B	C	D	A	B	C	D
Aflatoxin	Peak height [in mV]							
AfB_1	128	100	137	110	148	138	176	162
AfB_2	79	66	87	96	90	94	120	114
AfG_1	102	89	105	100	117	128	127	126
AfG_2	90	83	95	99	110	106	123	122
Aflatoxin	Percentage compared to Linomat							
AfB_1	86	72	78	78	-	-	-	-
AfB_2	87	70	76	84	-	-	-	-
AfG_1	87	70	83	79	-	-	-	-
AfG_2	82	78	77	81	-	-	-	-

Table 2.19: Differences in results from classical spotting and spraying. A = MeOH; B = acetone:hexane (15+85); C = MeOH:acetone:hexane (5+5+90); D = acetone:cyclohexane (10+90)

The reason for this is thought to be that the solvent penetrates the TLC layer directly using the classical spotting, while on the other hand they start to evaporate even before the analyte reaches the TLC plate, when sprayed.

Thus, a higher concentration of the analyte in the upper layers of the TLC-plate can be expected. This assumption is further supported by the fact that the biggest difference occured with the solution that evaporated fastest (Solution B), while MeOH resulted in the smallest difference.

It seems that due to the different experimental design (spotting and spraying was performed on different plates) [115] this phenomenon remained uncovered, since no direct comparison was achievable this way [118].

Concerning the precision for both application methods no differences were found for solutions spotted and sprayed with MeOH-acetone-hexane, which supports the previously found data.

Additional experiments were carried out to investigate the effect of different spot or band sizes on densitometric signals. This parameter was thought to be important, in case aflatoxins were spotted manually at different application speeds and thus might differ *even* slightly. Therefore equal amounts of aflatoxins were sprayed as bands of different sizes from 2 mm - 8 mm on the TLC-plate. This allowed the investigation of any tendencies or differences that might occur. Aflatoxin spots were pre-developed with MeOH to flatten bands and spots. No relevant difference or tendency was observed, provided that the area scanned recorded the total amount of aflatoxin applied[15].

Thin-layer Separation of Aflatoxins

TLC-plate material Since the previous immunoaffinity column clean-up delivered highly purified aflatoxins, the aim was to achieve a sufficient TLC separation with a single one-dimensional development. Several TLC-materials have been investigated in the past [119–122] while despite certain advantages for polyamide [122], the overall performance of silica gel succeeded in most applications. Generally silica gel TLC plates have appropriate optical properties for the fluorescence detection of the aflatoxins, are easy to use and are fairly inexpensive. In addition, there are performance studies for aflatoxin determination on different silica gel materials [123, 124] and on fluorometric measurements in general [125]. The authors concluded that HPTLC material offers the best performance, while the quality of silica gel plates is an important parameter for precise fluorometric measurements. However, the primary strategy was to evaluate *standard* TLC plates, since HPTLC material is costly and was only considered if separations were not sufficient.

Mobile phase composition During mobile phase testing, special attention was again drawn to the absence of chlorinated solvents and those with a

[15]Scanned band size was 12 mm for all spots

low toxicity. Nevertheless, this mobile phase shall allow the separation of all four aflatoxins in a one-dimensional run. This was considered to be essential, to justify the use of IAC in combination with TLC.

In previous work several mobile phase compositions have been described [126–129] [130–134]. However, only one mobile phase based on a mixture of diethyl ether, MeOH and water [133] was confirmed to be promising. This mobile phase was simply modified by substituting the highly volatile and peroxide susceptible diethyl ether with the significantly more stable *tert*-butyl-methyl-ether (*t*-BME). In conclusion, a mobile phase composed of *t*-BME-MeOH-water (480+15+5 [v/v/v]) was found to deliver the same and sufficient results as the diethyl candidate, while t-BME even offers a (more favourable) higher boiling point.

Nevertheless, the evaporation of the mobile phase after TLC development is still very rapid and requires no vacuum, or heat. These features offer an easy handling of the mobile phase at room temperature. All four aflatoxins were sufficiently separated in a single run with R_f-values of 0.40 (AfB_1), 0.35 (AfB_2), 0.29 (AfG_1), and 0.25 (AfG_2) using an unlined and unequilibrated tank (Figure 2.28). Therefore the simultaneous application of several samples and standards on one TLC-plate is possible, while classical clean-up procedures[16] require a two dimensional TLC-separation for many matrices with interfering compounds [107, 108]. This requirement drastically limits the number of samples that can be applied on a single plate.

Additional experiments for the robustness of the mobile phase were performed, by repeatedly use of the mobile phase. The main effect was a shift to increased R_f-values. However, the separation of all four aflatoxins was still satisfying and showed the ability to reuse the mobile phase several times.

Fluorescence amplification after TLC development The fluorescence intensity of an organic substance can be quenched or amplified by certain parameters such a pH and other chemical or physical environmental changes. In HPLC the fluorescence of aflatoxins is known to be amplified by chemical derivatization [79, 135] or by additives in the mobile phase such as the addition of cyclodextrines [102, 136]. In the latter case the amplification is of a physical nature. Similar to this effect, the amplification of fluorescence for TLC has been described in the literature [137] by simple means of dipping or spraying the TLC-plates in paraffin solutions. An interesting observation was found to be the amplification of aflatoxins by free fatty acids (as a result of matrix interference), causing recoveries far above 100% [138].

In order to investigate the fluorescence amplification, a TLC-plate was

[16]Solid phase extraction

scanned twice. Once prior spraying on once and after spraying half the plate with a 10% paraffin solution in n-hexane. The sprayed spots resulted in an amplified fluorescence about twice as high as prior spraying.

However irregular spraying can be an additional source of error. Therefore spraying procedures were only considered when spots (close to the LOD) would result in poor fluorescence for visual judgement.

In-House Validation of the TLC-method

Parameters for the in-house validation were mainly taken from the criteria laid down by the European Standardization Committee (CEN) [51]. All figures were calculated with a software package for the validation of analytical methods [139].

There are several approaches (algorithms) for the determination of performance characteristics from analytical data. For the determination of in-house characteristics, the parameters LOD and LOQ were calculated from the 95% confidence interval of the calibration curve, while the recovery was determined from the slope thereof.

Densitometry In a first step the calibration curves for all four aflatoxins were established with standards in a range reflecting contamination levels of 0.1 μg/kg - 1 μg/kg for each toxin. As an example, the calibration curve for aflatoxin B_1 is shown in Figure 2.31, while the densitograms are given in Figure 2.32. These calibration results (with standard solutions) will be discussed in detail in Chapter 2.4.4, where different densitometers will be compared.

However, the LOD was found to range from 100 pg to 200 pg for all four aflatoxins, while the LOQ was 200 pg to 300 pg. Such an amount reflects a contamination level 0.2 μg/kg to 0.3 μg/kg aflatoxin for the above described TLC procedures, showing that this method should be suitable for the determination of aflatoxins at the current legislative level.

Additional calibration curves with fortified samples were produced for all matrices. Such a procedure considers all procedures of the method including sample preparation. Therefore a representative parameter for the *overall* method performance can be obtained. Samples of paprika powder, peanut butter and pistachios (blank materials) which reflect the most relevant and demanding matrices in aflatoxin analysis were spiked at levels of 1 μg/kg - 4 μg/kg, analysed and the results were plotted against the spiked level.

The resulting slopes of these calibration curves are a figure for the recovery of the method, while from the confidence interval of the calibration curve (95%) the LOD and the LOQ were calculated (Table 2.20). These

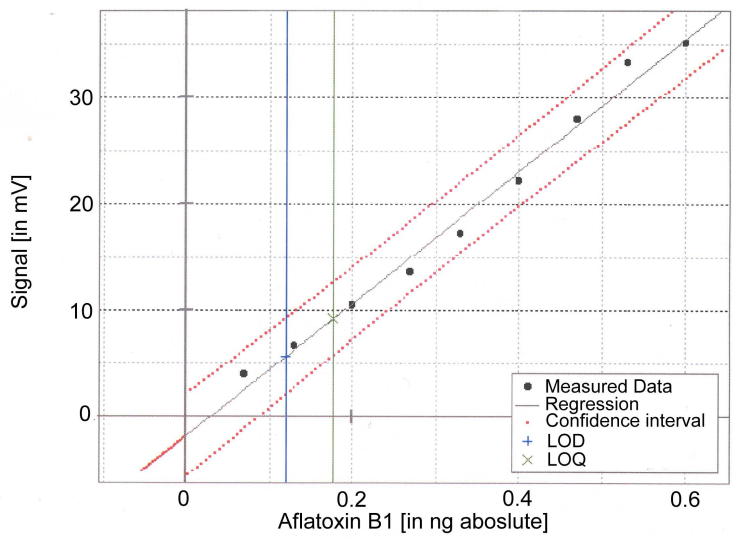


Figure 2.31: Calibration curve for aflatoxin B_1

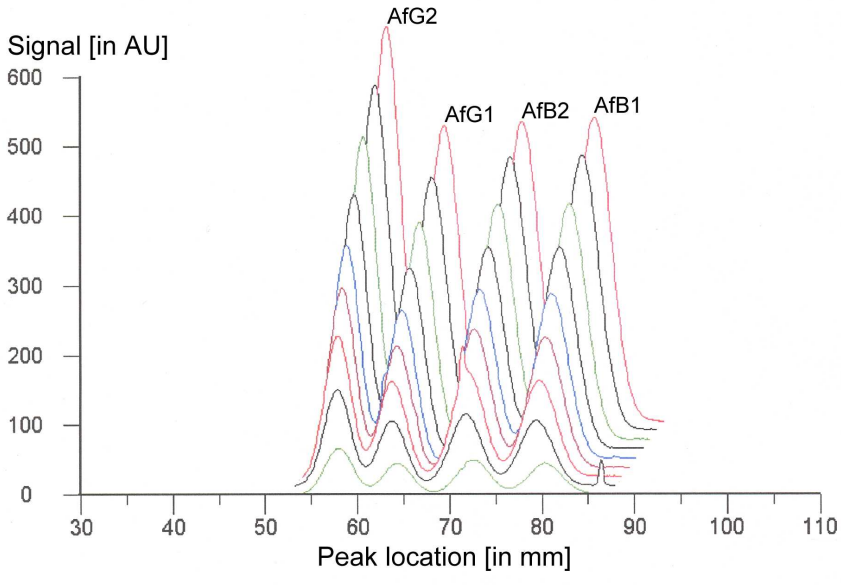


Figure 2.32: Densitograms of aflatoxins after TLC separation

calculations were performed with a software for the validation of analytical methods [139].

Matrix	Analyte	LOD	LOQ	Recovery	RSD
Peanut butter	AfB_1	0.6μg/kg	1.5μg/kg	85%	6.0%
"	AfB_2	0.2μg/kg	0.6μg/kg	87%	2.6%
"	AfG_1	0.6μg/kg	1.7μg/kg	82%	6.7%
"	AfG_2	0.4μg/kg	1.4μg/kg	78%	5.4%
Paprika	AfB_1	0.4μg/kg	1.2μg/kg	85%	4.9%
"	AfB_2	0.3μg/kg	0.9μg/kg	87%	3.6%
"	AfG_1	0.5μg/kg	1.6μg/kg	84%	6.5%
"	AfG_2	0.7μg/kg	2.2μg/kg	76%	8.9%
Pistachios	AfB_1	0.2μg/kg	0.5μg/kg	82%	1.9%
"	AfB_2	0.1μg/kg	0.4μg/kg	87%	1.4%
"	AfG_1	0.3μg/kg	0.8μg/kg	81%	3.4%
"	AfG_2	0.3μg/kg	0.8μg/kg	83%	3.3%

Table 2.20: Results of the TLC method Characterization

Visual judgement Parallel to the densitometric measurements, laboratory staff members were asked to visually quantify the peaks. Thus, for the determination of the LOD, decreasing amounts of aflatoxins in the range of 1.0 ng - 0.1 ng had to be identified on a developed TLC-plate with a UV-light lamp (366 nm).

The test panel had to score the number of spots that could be identified. As little as 0.5 ng of aflatoxins B_1 and B_2 and as little as 0.3 ng of AfG_1 and AfG_2 could be identified by all panel members (n=6), while individuals were able to identify even amounts of 0.1 ng AfG_1 and AfG_2.

Additional triangle, ranking and the duo-trio tests were performed with AfB_1 only, since it is the main contaminant in food and its blue fluorescence (together with that of AfB_2) is far more difficult to visualise than the bright-green fluorescence of AfG_1 and AfG_2 at same levels.

Tables 2.21 and 2.22 give an overview on the results of these tests and the amounts found by densitometry. As can be seen, it was possible for the panel members to distinguish between AfB_1 amounts of 0.5 ng, 1 ng and 2 ng. This demonstrates that the visual determination under UV-light allows semi-quantitative judgements at a level of 1 ng[17] and above.

[17]Equivalent to a contamination level of 1 μg/kg

Test	**Spot 1**	**Spot 2**	**Spot 3**	**False**	**Correct**	**Errors** [%]
Triangle 1	1	2	1	0	6	0
Triangle 2	1	0.5	1	0	6	0
Triangle 3	2	2	0.5	0	6	0
Ranking 1	0.5	2	1	0	6	0
Ranking 2	2	0.5	1	0	6	0
Ranking 3	2	1	0.5	0	6	0

Table 2.21: Triangle and ranking test at levels of 0.5 ng, 1 ng and 2 ng

The triangle and ranking test consisted out of three sets on one TLC plate. The panel only had to identify the aflatoxin B_1 spots.

Spot 1	**Spot 2**	**Spot 3**	**Test design**	**Answers**	**Errors** [in %]
1 ng	1 ng	2 ng	Triangle	(1-1-2)=1 (2-1-3)=4	83 [17]
80 mV	75 mV	157 mV		(1-2-3)=1	
1 ng	2 ng	2 ng	Triangle	(1-2-2)=4 (1-3-2)=1	33
73 mV	158 mV	160 mV		(2-2-1)=1	
0.5 ng	1 ng	2 ng	Ranking	(1-2-3)=5	17
36 mV	70 mV	124 mV		(1-2-2)=1	

Table 2.22: Duo-Trio test at levels of 0.5 ng, 1 ng and 2 ng

For the duo-trio test the participants had to judge the spots compared to spot #2. Participants did not know if a difference existed. In addition, the visual determination spots were scanned (figures in mV) to identify any objective differences in fluorescence intensity. In addition, the ranking order and number of the corresponding answers from the panel (n=6) are given.

A final evaluation was made in which the panel had to judge a series of unknown aflatoxin spots on a TLC plate. Participants were asked to compare adjoined aflatoxins and to report if they were able to determine a difference between two spots (yes/no) and what the order of brightness was, while no further information was given. The TLC-plate to investigate contained 9 sets of spots (5 fortified samples and 4 standards) in the range of 0 - 4μg/kg.

Furthermore, for confirmation the TLC-plate was scanned densitometrically and the results were compared with the visual outcome.

Spot#	**x** [ng]	**p** [ng]	**Δ**	**Correct answers**	**Answers (n=9)**		
					x<p	x>p	x=p
1	3.2	4.0	21%	56%	5	-	4
2	4.0	2.4	41%	100%	-	9	-
3	2.4	3.0	21%	100%	9	-	-
4	3.0	2.0	33%	100%	-	9	-
5	2.0	1.6	19%	22%	1	2	6
6	1.6	0.6	61%	100%	-	9	-
7	0.6	1.0	36%	100%	9	-	-
8	1.0	0	100%	100%	-	9	-
9	0.0	-	-	-	-	-	-

Table 2.23: Result of the visual aflatoxin determination

As shown in Table 2.23 it was possible to determine differences between the separated and interference free aflatoxin spots from extracted pistachios, if spots differed more than 20%. Similar values were previously found by [140], who reported that differences of 20% can be determined by trained operators.

The x-value [ng] and the p-value [ng] are the amounts of aflatoxin spotted, next to each other. These spots were compared. Spot numbers 2, 4, 5, and 8 (italic figures) are spotted standard solutions. The remaining spot reflect aflatoxins from fortified pistachio samples. The values from the fortified samples were determined by densitometry.

As can be seen, the fortified samples could be successfully ranked between corresponding standard levels, even though the panel members were laboratory co-workers with little or no experience in visual judgement. Therefore even better results would be expected with trained staff.

2.3 Validation by Collaborative Trial Studies

2.3.1 Requirements for Validated Methods

Performance Criteria of Validated Methods

Any method that is designed to be validated and adopted by *CEN* or *AOAC Int* as an official method must fulfill certain precision criteria. In addition,

it must be feasible to quantify the analyte (aflatoxins) at the selected target level. In the case of aflatoxins the Commission Directive on methods for sampling and analysis of food certain contaminants [141] lays down the precision for analysis methods. This fact clearly states that a method which will be used for monitoring contaminants according to legislation will have to comply with international guidelines in the conduct of statistical evaluation [142, 143] and in the method performance thereof [51, 96]. Therefore throughout this work described HPLC-methods were first in-house tested and subsequently subject to an international collaborative trial according to the statutes of *AOAC Int.*

Production of Homogeneous Test Materials

Due to the fact that this study was concerned with considerably low contamination levels of aflatoxins in the various food matrices, particular care was taken in the preparation of test materials for the collaborative trial. The procurement comprised the grinding of the raw materials to very small particle sizes followed by an extensive mixing and blending. In addition to the demonstration of bulk homogeneity prior to packing, the inter-unit homogeneity of the packed materials was shown after packing and before undertaking the foreseen collaborative trial by the means of the statistical ANOVA. After packing, all materials[18] were subsequently heat treated at 80°C for approximately 20 minutes and stored at -18°C in order to avoid growth of microorganisms or aflatoxin degradation. The test for *container*-homogeneity indicated that sufficient homogeneity was achieved in all packed materials. Table 2.24 shows the results from this homogeneity testing.

Aflatoxin degradation in fig paste In the case of fig paste an unexpected effect occurred which could be traced back to the heat treatment during the autoclave process (80°C for 20 minutes). Aqueous MeOH extracts of these test materials appeared to be different in colour, thus indicating that the material was not homogeneous in all its components, even though bulk homogeneity for aflatoxins was obtained. The further aflatoxin determination (container) revealed the same results as the observed colour differences. This led to further investigation. The maximum absorption of the inhomogeneous extracts was found to be at 410 nm. For a ranking test, the brightest extract was taken as a reference, while all other extracts were measured for absorption at 410 nm against it. As shown in Figure 2.33 a negative correlation between the measured aflatoxin content and the absorption of the extract was found.

[18] Except fig paste materials

Material	**Target** AfB_1 μg/kg	**Achieved** AfB_1 μg/kg	**F-value** ($F_{crit} = 3.02_{p=0.05}$)
Pistachio paste	0	0.2	-
	1	0.8±0.06	0.4
	2	1.7±0.27	1.5
	4	3.5±0.15	0.1
Peanut butter	0	<0.1	-
	1	1.0±0.17	1.0
	2	1.6±0.27	1.0
	4	3.6±0.47	0.6
Fig paste	0	0.3±0.05	1.9
	1	1.3±0.04	0.1
	2	2.0±0.97	0.3
	4	3.3±0.33	1.2
Paprika powder	0	<0.1	-
	1	1.1±0.15	1.0
	2	1.8±0.14	0.3
	4	4.0±0.36	0.4
Infant formula	0	<0.05	-
	0.05	0.09±0.02	1.6
	0.1	0.13±0.02	0.7
	0.2	0.21±0.04	0.8
Animal feed	0	0.09±0.004	-
	0.5	0.68±0.02	0.7
	1	1.03±0.01	0.2
	5	5.21±0.10	0.6

Table 2.24: Results from the various homogeneity studys on AfB_1

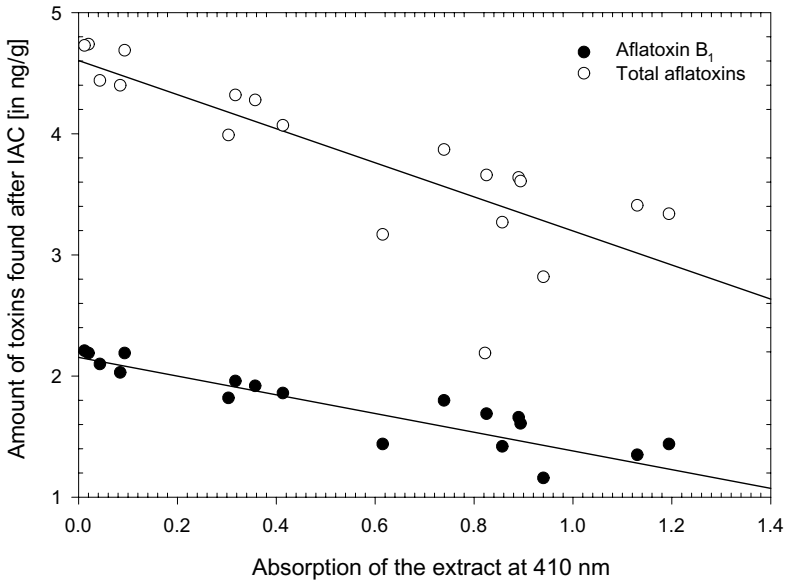


Figure 2.33: Correlation between extract colour and aflatoxin content of fig extracts after heat treatment

The darkening of extracts was explained to be due to the Maillard-Reaction (so called »*non enzymatic browning*«). Apparently the correlation indicated that the measurement of aflatoxins will be affected by matrices that undergo Maillard-Reaction (e.g. during storage or treatment). The exact mechanism, which caused this apparent loss of aflatoxins needs further investigation and it is still unclear whether both effects (lower aflatoxin values and Maillard-Reaction) are linked, or just correlate, whether the aflatoxins will be *masked* or undergo chemical degradation to non toxic metabolites in the food matrix. The fact that (measured) aflatoxin levels decrease during heat treatment or chemical treatment [8–10, 144] is known and has been investigated for the detoxification of feeding stuffs [144], however the reported conditions were far above 100^oC. Furthermore, correlations of *browning effects* of sample extracts or HMF content and measured aflatoxin concentration has not been described before.

Due to this resulting inhomogeneity of the fig material, it was prepared twice. All results given in this work concerned the second batch of non heat-treated material, which appeared to be stable and homogeneous.

2.3.2 Organization of the Collaborative Trial

The participating laboratories from the different countries represented a cross-section of national research organizations, food control authorities, and food industry affiliations. Prior to the trial each participant received a practice sample which comprised blank material and a calibrant solution for spiking. A pre-collaborative trial workshop was organized by myself in collaboration with other partners of the project where any problems experienced with the analysis of the practice sample were discussed, and the details and organization of the trial were outlined by the co-ordinators.

Each of the collaborative trial participants received:

- Eight coded samples of each matrix (blind duplicates at four content levels) plus four units labelled »blank« (for spiking).
- One amber vial marked »Aflatoxin calibrant« containing a mixture of aflatoxins B_1, B_2, G_1 and G_2, which was to be employed as the (mixed) calibrant aflatoxin solution described in the method.
- Two amber vials marked »Spike solution A« and »Spike solution B« to be used for spike recovery determinations.
- A sufficient amount of immunoaffinity columns (with a minimum of 2 spares) for the clean-up of material extracts.

- A copy of the method.
- A report form for analytical data as well as reporting any criticisms and suggestions.
- A »Collaborative Study Materials Receipt« form.

Each participant was required to prepare one extract from each material, perform the clean-up using one immunoaffinity column and analyse the extracts by HPLC. Additionally each participant was asked to spike four indicated *blank* materials of each matrix at two different levels with *Solution A* and *Solution B* which contained aflatoxins at concentrations unknown to participants. After adding the spike solution, participants were instructed to mix by shaking and allow the sample to stand for at least 30 minutes prior to extraction.

In case different matrices had to be analysed, participants were advised to analyse different matrices on separate days. This would mean analysing a batch of 12 samples (8 coded plus 4 spike samples per matrix) on separate days. Participants were instructed that samples should be analysed in the numerical sequence of the sample codes (nested design).

2.3.3 Results of the Collaborative Trial

Collaborative Trial Results for Paprika Powder, Fig Paste, Peanut Butter and Pistachio Paste

All 16 collaborators who received test samples completed the study. All data submitted for the study for the four commodities are presented in the Annex (Tables 5.1 to 5.16). Each table is sub-divided presenting individual results for aflatoxins B_1, B_2, G_1, G_2 and total aflatoxins. The data are given as individual pairs of results for each participant (identified as A to P). Blank samples (identified as samples *c*) were spiked with 1.0 μg/kg and 4.0 μg/kg of aflatoxins B_1 and G_1 and 0.2 μg/kg and 0.8 μg/kg for aflatoxins B_2 and G_2 (identified as samples *a* and *b* and giving corresponding levels for total aflatoxins of 2.4 μg/kg and 9.6 μg/kg respectively). Samples *d*, *e* and *f* are blind duplicates for naturally contaminated materials in each case.

Precision estimates were obtained using the one-way analysis of variance approach according to the IUPAC Harmonized Protocol [52]. Details of the food matrices, the average analyte concentration, the standard deviations

for repeatability (s_r) and reproducibility (S_R), the relative standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories and the percentage recovery are presented in Tables 2.25 to 2.28 for aflatoxins B_1 and total aflatoxins respectively. The collaborative trial results had previously been examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively [52]. Pairs of results that were identified as outliers are given in *slanted** numbers in Tables 5.1 to 5.16.

Regarding the results for aflatoxin B_1 and for total aflatoxins (excluding data for blank materials) the maximum numbers of outliers identified was 4 laboratories, giving acceptable data from 12 to 16 laboratories. Due to differences in reporting limits for *not detectable* amounts, the results for *blank* materials in the cases of peanut butter and paprika powder were not analysed statistically. However the results clearly indicated that all participants could identify the blank pairs of samples as not containing detectable aflatoxins or containing levels which were detectable but close to measurable limits. The results for the *blank* samples of pistachio and fig pastes showed that both samples contained low but measurable amounts of aflatoxins. The average levels of aflatoxin B_1 in the pistachio and fig pastes were found to be 0.13 μg/kg and 0.32 μg/kg respectively (corresponding to 0.2 μg/kg and 0.6 μg/kg for total aflatoxins respectively). In the case of aflatoxins G_1 and G_2 in pistachio paste, the results for the three naturally contaminated samples (*d*, *e* and *f*) contained levels that were below the limits of detection (3x baseline noise), and therefore it was not possible to undertake statistical analysis in this instance.

The precision data is shown in Tables 2.25 to 2.28. Based on results for spiked samples (blind pairs at two levels) as well as naturally contaminated samples (blind pairs at three levels) the relative standard deviation for repeatability (RSD_r) ranged from 4.6% to 23.3% for total aflatoxins and from 3.1% to 20.0% for aflatoxin B_1. The relative standard deviation for reproducibility (RSD_R) ranged from 14.1% to 34.2% for total aflatoxins, and from 9.1% to 32.2% for aflatoxin B_1. This precision data is comparable with those from other reference methods [68], while the range of food items covers all important products at the relevant working level.

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	13	15	14	14
Number of outliers	1	3	1	2	2
Number of accepted results	15	13	15	14	14
Mean value [in μg/kg]	0.87	3.65	0.80	1.52	3.40
Repeatability standard deviation s_r [in μg/kg]	0.09	0.11	0.05	0.10	0.13
Repeatability relative standard deviation RSD_r [in %]	10	3	6	6	4
Repeatability limit $r(r{=}2.8{*}s_r)$ [in μg/kg]	0.25	0.31	0.14	0.28	0.36
Reproducibility standard deviation s_R [in μg/kg]	0.16	0.66	0.26	0.22	0.65
Reproducibility relative standard deviation RSD_R [in %]	19	18	32	14	19
Reproducibility limit $R[R{=}2.8{*}s_R]$ [in μg/kg]	0.45	1.85	0.73	0.62	1.82
Recovery [in %]	87	91	-	-	-
Parameter	**Total aflatoxins**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	15	15	13	14
Number of outliers	1	1	1	3	2
Number of accepted results	15	15	15	13	14
Mean value [in μg/kg]	1.9	7.9	1.3	2.2	5.0
Repeatability standard deviation s_r [in μg/kg]	0.26	0.67	0.08	0.16	0.23
Repeatability relative standard deviation RSD_r [in %]	13	9	6	7	5
Repeatability limit r $(r{=}2.8{*}s_r)$ [in μg/kg]	0.73	1.88	0.22	0.45	0.64
Reproducibility standard deviation s_R [in μg/kg]	0.35	1.76	0.46	0.32	0.96
Reproducibility relative standard deviation RSD_R [in %]	18	22	34	14	19
Reproducibility limit R $[R{=}2.8{*}s_R]$ [in μg/kg]	0.98	4.93	1.29	0.90	2.69
Recovery [in %]	81	82	-	-	-

Table 2.25: Precision data for aflatoxin B_1 in peanut butter

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	12	13	15	14
Number of outliers	1	4	3	1	2
Number of accepted results	15	12	13	15	14
Mean value [in μg/kg]	0.94	3.29	0.74	1.54	2.93
Repeatability standard deviation s_r [in μg/kg]	0.13	0.13	0.08	0.27	0.59
Repeatability relative standard deviation RSD_r [in %]	14	4	11	18	20
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	0.36	0.36	0.22	0.76	1.65
Reproducibility standard deviation s_R [in μg/kg]	0.15	1.02	0.12	0.36	0.61
Reproducibility relative standard deviation RSD_R [in %]	16	31	17	23	21
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	0.42	2.86	0.34	1.01	1.71
Recovery [in %]	94	82	-	-	-
Parameter	**Total aflatoxins**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	14	14	13	15	14
Number of outliers	2	2	3	1	2
Number of accepted results	14	14	13	15	14
Mean value [in μg/kg]	2.0	7.8	0.8	1.7	3.3
Repeatability standard deviation s_r [in μg/kg]	0.24	1.82	0.10	0.31	0.66
Repeatability relative standard deviation RSD_r [in %]	12	23	12	18	20
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	0.67	5.10	0.28	0.87	1.85
Reproducibility standard deviation s_R [in μg/kg]	0.36	1.82	0.17	0.42	0.72
Reproducibility relative standard deviation RSD_R [in %]	18	23	21	24	22
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	1.01	5.1	0.48	1.18	2.02
Recovery [in %]	83	81	-	-	-

Table 2.26: Precision data for pistachio paste

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	15	16	14	16
Number of outliers	1	1	0	2	0
Number of accepted results	15	15	16	14	16
Mean value [in μg/kg]	1.10	3.60	1.32	2.07	2.55
Repeatability standard deviation s_r [in μg/kg]	0.18	0.39	0.12	0.12	0.41
Repeatability relative standard deviation RSD_r [in %]	17	11	10	6	16
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	0.5	1.09	0.34	0.34	1.15
Reproducibility standard deviation s_R [in μg/kg]	0.21	0.46	0.30	0.31	0.73
Reproducibility relative standard deviation RSD_R [in %]	19	13	23	15	29
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	0.59	1.29	0.84	0.87	2.04
Recovery [in %]	109	90	-	-	-
Parameter	**Total aflatoxins**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	15	15	16	16	16
Number of outliers	1	1	0	0	0
Number of accepted results	15	15	16	16	16
Mean value [in μg/kg]	2.2	7.8	2.8	3.8	5.2
Repeatability standard deviation s_r, [in μg/kg]	0.40	1.01	0.25	0.44	0.90
Repeatability relative standard deviation RSD_r [in %]	18	13	9	12	17
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	1.12	2.83	0.7	1.23	2.52
Reproducibility standard deviation s_R, [in μg/kg]	0.73	1.28	0.80	1.03	1.56
Reproducibility relative standard deviation RSD_R [in %]	32	17	28	29	30
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	2.04	3.58	2.24	2.88	4.37
Recovery [in %]	92	81	-	-	-

Table 2.27: Precision data for fig paste

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	14	15	15	15	14
Number of outliers	2	1	1	1	2
Number of accepted results	14	15	15	15	14
Mean value [in μg/kg]	0.86	3.41	0.84	1.39	3.02
Repeatability standard deviation s_r [in μg/kg]	0.05	0.18	0.12	0.14	0.13
Repeatability relative standard deviation RSD_r [in %]	6	5	14	10	4
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	0.14	0.5	0.34	0.39	0.36
Reproducibility standard deviation s_R [in μg/kg]	0.09	0.35	0.16	0.24	0.28
Reproducibility relative standard deviation RSD_R [in %]	10	10	19	17	9
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	0.25	0.98	0.45	0.67	0.78
Recovery [in %]	86	85	-	-	-
Parameter	**Total aflatoxins**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	13	15	16	16	14
Number of outliers	3	1	0	0	2
Number of accepted results	13	15	16	16	14
Mean value [in μg/kg]	1.7	7.1	0.9	2.0	4.5
Repeatability standard deviation s_r [in μg/kg]	0.11	0.72	0.16	0.23	0.22
Repeatability relative standard deviation RSD_r [in %]	6	10	17	12	5
Repeatability limit r (r=2.8$*s_r$) [in μg/kg]	0.31	2.02	0.45	0.64	0.62
Reproducibility standard deviation s_R [in μg/kg]	0.34	1.01	0.31	0.55	0.66
Reproducibility relative standard deviation RSD_R [in %]	20	14	34	28	15
Reproducibility limit R [R=2.8$*s_R$] [in μg/kg]	0.95	2.83	0.87	1.54	1.85
Recovery [in %]	71	74	-	-	-

Table 2.28: Precision data for paprika powder

Although the method described here is only recommended for application at levels of aflatoxin B_1 at greater than 1 μg/kg and for total aflatoxins at greater than 2.4 μg/kg, there is evidence from the *blank* matrices that contained low levels of aflatoxins that the method in fact gives satisfactory performance at lower levels. Thus, for pistachio and fig pastes containing levels of 0.1 μg/kg and 0.3 μg/kg aflatoxin B_1 respectively (and corresponding total aflatoxin levels of 0.2 μg/kg and 0.6 μg/kg respectively) RSD_r's of 19.2% and 24.5% and RSD_R's of 49.5% and 30.6% were obtained for aflatoxin B_1 and RSD_r's of 53.4% and 43.3% and RSD_R's of 58.2% and 52.9% were obtained for total aflatoxins. Although these precision values are higher than those at detection levels at which the method is being proposed, the HORRAT values are nevertheless still below 2.0 and only in one instance slightly exceed 1.0.

Thus in principle the method could be claimed as operable with acceptable performance characteristics at levels as low as 0.1 μg/kg for aflatoxin B_1 and 0.2 μg/kg for total aflatoxins in pistachio paste. The values for recoveries of aflatoxin B_1 derived from the spiked samples were found to range from 82% to 109% and for total aflatoxins to range from 71% to 92% (Tables 2.25 to 2.28).

The lowest recoveries were obtained for paprika powder, and the highest values for fig paste where the *blank* material used for spiking contained low levels of aflatoxins. When the average level of aflatoxin B_1 of 0.3 μg/kg in the *blank* fig paste is deducted from measured values after spiking, the recovery values decreased from 109% to 78% and from 90% to 82%. Similarly when the measured level of total aflatoxins of 0.6 μg/kg in the *blank* fig paste was deducted, the recoveries decreased from 92% to 67% and from 81% to 75%. Recoveries for aflatoxins B_2, G_1 and G_2 ranged from 58% to 123% with the lowest recoveries again being for paprika powder and the high recoveries being for fig paste where, if *blank* background levels were subtracted, decrease from 123% to 75%. As the method is envisaged as being applied to determine either or both aflatoxin B_1 and total aflatoxins, the recoveries were deemed as acceptable.

The acceptability of the precision characteristics of the method were assessed on the basis of the HORRAT values [145] which compare the RSD_R at the various levels and in the various matrices with those values predicted from collaborative trial studies taken from published literature. When outliers were excluded, the HORRAT values for aflatoxin B_1 ranged from 0.3 to 0.7 for peanut butter, from 0.4 to 0.8 for pistachio paste, from 0.3 to 0.7 for fig paste and from 0.2 to 0.4 for paprika powder. The HORRAT values for aflatoxin B_2, G_1 and G_2 were generally of the same order as those

for aflatoxin B_1 and only in one case (for aflatoxin G2 in a sample of fig paste) were the HORRAT values >1. For total aflatoxins the HORRAT values ranged from 0.3 to 0.8 for peanut butter, from 0.4 to 0.7 for pistachio paste, from 0.5 to 0.8 for fig paste and from 0.4 to 0.7 for paprika powder. In any event all HORRAT values were <2.0 which is taken as indicating an acceptable precision, and were better than or comparable to values reported in the AOAC-IUPAC official first action method [146] notwithstanding the low levels of aflatoxin contamination in this instance.

Collaborative Trial Results for Infant Formula

Fourteen laboratories participated in the study and all laboratories completed the study. The full set of single results for the trial is given in the Annex (Table 5.17) as individual pairs for each sample and for each participant (identified as 1 to 14).

The collaborative trial results were examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively [52]. Pairs of results that were identified as outliers are given in *italics** in Table 5.17.

Blank samples (identified as sample *c*) were spiked with 0.1 μg/kg and 0.2 μg/kg of aflatoxin B_1 as blind duplicates (identified as samples *a* and *b* respectively). Samples *d*, *e* and *f* are blind duplicates for naturally contaminated materials in each case.

Precision estimates were obtained using one-way analysis of variance approach according to the IUPAC Harmonized Protocol [52]. Details of the average aflatoxin B_1 contents of the various baby food test samples, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the HORRAT values and the mean percentage recovery are presented in Table 2.29. For the results for aflatoxin B_1 given in Table 5.17 (excluding the data for blank materials) the maximum numbers of outliers identified was 3 laboratories, giving acceptable data ranging from 11 to 14 laboratories.

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	11	14	14	11	13
Number of outliers	3	0	0	3	1
Number of accepted results	11	14	14	11	13
Mean value [in μg/kg]	0.10	0.18	0.07	0.09	0.17
Repeatability standard deviation s_r [in μg/kg]	0.004	0.024	0.010	0.007	0.021
Repeatability relative standard deviation RSD_r [in %]	3.5	13	14	8	12
Repeatability limit $r(r{=}2.8{*}s_r)$ [in μg/kg]	0.011	0.067	0.028	0.020	0.059
Reproducibility standard deviation s_R [in μg/kg]	0.012	0.042	0.017	0.008	0.039
Reproducibility relative standard deviation RSD_R [in %]	12	23	23	9	23
Reproducibility limit $R[R{=}2.8{*}s_R]$ [in μg/kg]	0.034	0.118	0.048	0.022	0.109
Recovery [in %]	101	92	-	-	-

Table 2.29: Precision Data for aflatoxin B_1 in infant formula

Due to differences in reporting limits for *not detectable* amounts, the results for *blank* materials were not analysed statistically. The results however clearly indicated that all participants could identify the blank pairs of samples as not containing detectable aflatoxin B_1 or containing a level which was detectable but close to their limit of determination. Based on results for spiked samples (blind pairs at two levels) as well as naturally contaminated samples (blind pairs at three levels) the relative standard deviation for repeatability (RSD_r) ranged from 3.5% to 14%, and the relative standard deviation for reproducibility (RSD_R) ranged from 9% to 23%.

The values for recoveries of aflatoxin B_1 derived from the spiked baby food samples were found to range from 101% to 92%. The acceptability of the precision characteristics of the method were assessed on the basis of the HORRAT values [145] which compare the RSD_R at the various levels with those values predicted from collaborative trial studies taken from the published literature. When outliers were excluded, the HORRAT value for

aflatoxin B_1 ranged from 0.l to 0.4, being significantly below 2.0 which indicates acceptable precision data.

Comments from collaborative trial participants Comments on the trial were made by 7 of the 14 participants. In all cases the participants regarded the method description as being adequate. For participant 9 where 2 pairs of results were rejected as being outliers, no comments were given. Participant 2 and participant 4 made no comments either. Participant 3 had prepared the standards in MeOH as solvent. Participant 12 expressed some doubt as to their results for spiked samples, but did not give a technical reason. However, the value for aflatoxin B_1 was found to be in compliance (i.e. this participant did not produce an outlier value for the spiked samples). Participant 13 again only used an injection volume of 200 μL, although no further comments were made or problems reported.

Collaborative Trial Results for Animal Feed

All 21 laboratories that received the test samples completed the study. The full set of results for this trial is given in the Annex (Table 5.18) as individual pairs of results for each participant (identified as 1 to 21). Blank samples (identified as sample *a*) were spiked with 1.2 μg/kg and 3.6 μg/kg of aflatoxin B_1 as blind duplicates. Samples *b*, *c*, and *d* were also blind duplicates but of naturally contaminated materials.

Precision estimates were obtained using one-way analysis of variance approach according to the AOAC Harmonized Protocol [52]. Details of the average aflatoxin B_1 contents of the various animal feeding stuff test samples, the standard deviations for repeatability (RSD_r) and reproducibility (RSD_R), the number of statistical outlier laboratories, the HORRAT values and the mean percentage recovery are presented in Table **??**. The collaborative trial results were examined for evidence of individual systematic error ($p<0.025$) using Cochran's and Grubbs tests progressively [52]. Pairs of results that were identified as outliers are given in *italic** numbers. For the results for aflatoxin B_1 given in Table 2.30 (excluding the data for blank materials) the maximum numbers of outliers identified was 3 laboratories, giving acceptable data ranging from 18 to 20 laboratories.

Number of laboratories	16				
Sample Set (nc = naturally contam.)	1	2	3(nc)	4(nc)	5(nc)
Parameter	**Aflatoxin B_1**				
Number of samples (duplicates)	1	1	1	1	1
Number of laboratories retained after eliminating outliers	11	14	14	11	13
Number of outliers	1	1	3	3	3
Number of accepted results	20	20	18	18	18
Mean value [in μg/kg]	1.33	3.89	0.54	0.87	4.19
Repeatability standard deviation s_r [in μg/kg]	0.08	0.25	0.04	0.08	0.26
Repeatability relative standard deviation RSD_r [in %]	5.9	6.4	7.2	8.7	6.2
Repeatability limit $r(r{=}2.8{*}s_r)$ [in μg/kg]	0.22	0.70	0.11	0.22	0.73
Reproducibility standard deviation s_R [in μg/kg]	0.72	1.87	0.27	0.47	2.30
Reproducibility relative standard deviation RSD_R [in %]	19.4	17.5	17.9	19.4	19.6
Reproducibility limit $R[R{=}2.8{*}s_R]$ [in μg/kg]	2.02	5.24	0.76	1.32	6.44
Recovery [in %]	111	108	-	-	-

Table 2.30: Precision Data for aflatoxin B_1 in animal feed

Comments from collaborative trial participants Comments were made by 13 of the 21 collaborative trial participants. Except for one case (participant 6), all other participants regarded the method description as being adequate. Results from participants 9, 11, 15 and 21 were rejected in some cases. All of these participants made comments, in the case of laboratories 9, 11 and 15 the reason for the outliers could not be clearly identified, while participant 21 indicated that the calibrants were prepared differently as given in the corresponding method section.

However when results of participant 21 were corrected for recovery they do not appear as outliers anymore. This strongly indicates a numerical (calculation) rather than a chemical reason for the outliers. Laboratories 2, 8, 9, 18, 20 and 21 indicated that the diluted extract remained turbid after filtration, whereas participant 2 re-filtered the solutions until clear. Participants 4, 9 and 18 used modified mobile phases to achieve better peak resolutions,

whereas participant 11 reported to have better signals with this method if the IAC eluate was evaporated and re-dissolved in the mobile phase. Laboratories 6, 12, 16, 18 and 19 made comments on the calibration, stating that the number of calibration points was exhaustive, while participant 16 had problems to get a sufficient signal for the lowest calibration point. Participant 8 noticed a significant PBPB-reagent degradation after two days (smaller peaks and higher background noise). Only the participant 6 used `option B` (all others used `option A`) due to poor signals. In one case, participant 15, it was stated that two test sample containers contained slightly less than 50 g, while one participant 18 used only a fraction of the containers for analysis.

Comparison of all Trial Results

A total of 28 different laboratories participated in at least one of the three trial studies, while several laboratories participated in more than one trial. As a result fifteen laboratories participated in two of the three trials, while 8 laboratories participated in all 3 trial studies (Table 2.31). The AfB_1 results from the fortification experiments (spiked samples) of these eight laboratories were extracted and compared for the reported recoveries. The aim of this procedure was to determine whether recoveries were random from trial to trial (for each laboratory) or if the recovery was systematically biased. The data analysis from fortified material was chosen, since these results were influenced by the overall procedures of the specific laboratory (collaborative trial method, handling of standard solutions and fortification procedure).

However previous to this between-trial comparison, the impact of this selective data extraction of the overall results was investigated. Therefore average recoveries (after elimination of outliers) of all laboratories and the one after extraction of the 8 laboratories were compared for each matrix and fortification level. As can be seen in Table 2.32 the obtained recoveries after the extraction of the 8 laboratories were not much different from the original recoveries.

The recoveries of the selected laboratories (Table 2.32) were plotted for each matrix and the two spiking levels (Figure 2.34). As can be seen, the matrix had a superior influence on the recovery than the spiking level. As expected, for most matrices the average recovery (mean of the 8 laboratories) was better for higher spiking levels (4 μg/kg), than those for lower spiking levels (1 μg/kg) for paprika powder, pistachio paste, peanut butter and fig paste, as well as infant formula (0.2 μg/kg and 0.1 μg/kg). Nevertheless these recovery differences were marginal and neglectable compared to the influence of the matrix/method.

Spiking levels (low and high) for animal feed were in a similar range

ID-1	ID-2	ID-3
-	5	N
7	-	-
3	**1**	**G**
-	2	B
-	10	O
19	-	-
8	-	-
14	-	-
-	-	J
5	-	-
1	**8**	**A**
6	**7**	**L**
12	**6**	**I**
17	**9**	**C**
9	-	-
2	-	E
15	-	-
4	**13**	**P**
18	-	-
20	-	-
-	11	H
10	12	-
13	**3**	**D**
16	-	-
11	**14**	**M**
-	-	K
-	4	F
21	-	-

Table 2.31: Codes of all trial participants: ID-1=animal feed trial; ID-2=infant formula trial; ID-3=pistachios-paprika-peanuts-fig trial. Laboratories that participated in all three trial are highlighted.

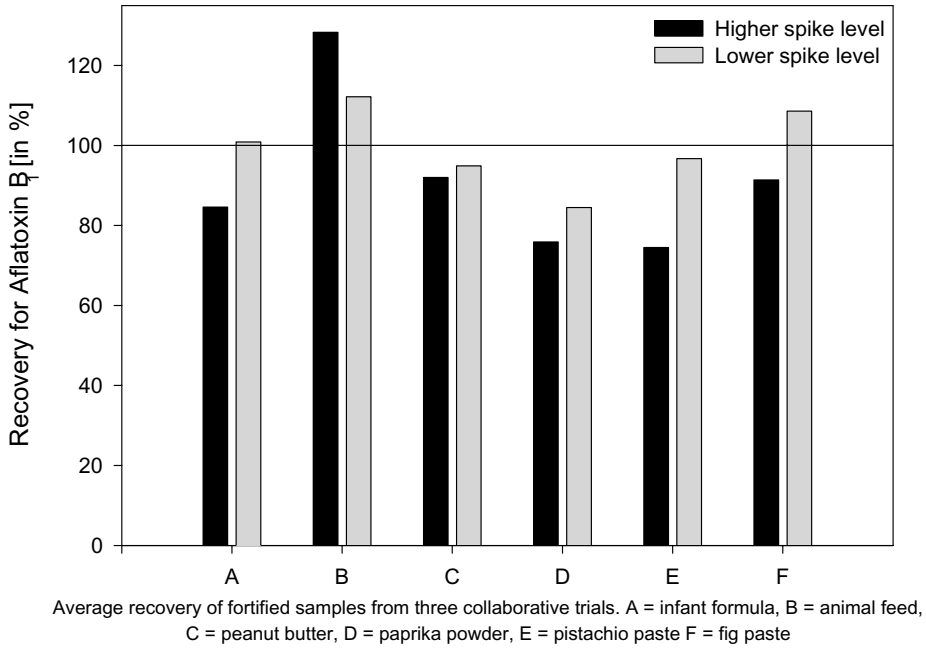


Figure 2.34: Recoveries of the different spike levels for each matrix

Analysed matrix	**Recovery**$_{\text{low}}$		**RSD**$_{\text{low}}$		**Recovery**$_{\text{high}}$		**RSD**$_{\text{high}}$	
	All	8	All	8	All	8	All	8
Infant formula	87%	80%	12%	11%	99%	99%	27%	25%
Animal feed	106%	108%	19%	13%	111%	112%	17%	14%
Peanut butter	91%	96%	18%	18%	87%	95%	18%	18%
Paprika Powder	85%	85%	10%	11%	86%	88%	10%	10%
Pistachio paste	89%	89%	16%	18%	94%	97%	6%	7%
Fig paste	90%	91%	20%	23%	108%	109%	13%	15%

Table 2.32: Comparison of the trial recoveries from »***All***« participants of each single trial and those »***8 ones***«, that participated in each of the 3 trials.

(1.2 μg/kg and 3.6 μg/kg), while in this case the recoveries obtained were significantly higher compared to the remaining matrices and recoveries for the lower spiking level were better in this case.

One explanation for the overall higher recoveries was that the animal feed method was based on the extraction with aqueous acetone (Chapter 2.1.1).

For further investigation, a comparison of the recoveries from each laboratory was compared with the mean recovery of the corresponding spiking level (Table 2.33) and several new observations could be drawn from the resulting data.

It could be shown that in the majority of cases the two corresponding spiking levels (*high* and *low*) of a single laboratory showed the same trend in the deviation from the mean recovery for a specific matrix (trial), while the deviation from matrix to matrix was more randomly distributed around the mean and gave no indication for a trend (Figures 2.35 and 2.36).

Since all fortification experiments (spiking) were made from separate and independent solutions, it can be concluded that this trend of *matching* recoveries for a specific matrix is an indicator for a laboratory and matrix dependent precision, that seems to be independent from the mean recovery.

Furthermore, the laboratories which produced several outliers (C and D) also reported at least 3 other recoveries that exceeded a 20% deviation for another or the same matrix (trial). On the other hand, some laboratories

Lab+Level	**Inf**	**Ani**	**Pea**	**Pap**	**Pis**	**Fig**
G_{high}	10.2	19.3	-18.6	-13.7	outl.	-9.4
G_{low}	-3.2	15.3	-17.9	-12.5	-16.2	-10.6
A_{high}	-4.1	3.4	-5.6	2.3	1.4	8.2
A_{low}	-0.4	-0.2	-4.5	1.8	-5.9	22.9
L_{high}	13.7	-6.8	-6.5	2.0	3.9	-4.9
L_{low}	-6.5	-9.7	-8.9	-1.5	4.3	10.4
I_{high}	13.4	12.2	-6.9	-8.1	6.4	7.2
I_{low}	16.0	9.9	-2.9	-8.5	14.3	16.9
C_{high}	-37.1	-8.1	34.5	-2.2	-1.8	outl.
C_{low}	outl.	10.3	28.1	4.0	-5.7	outl.
P_{high}	5.4	-3.7	5.3	7.6	outl.	-15.4
P_{low}	-4.5	-4.4	14.6	17.0	-11.2	5.4
D_{high}	15.4	-28.1	16.4	12.4	outl.	18.0
D_{low}	outl.	-27.9	8.6	outl.	23.8	-12.6
M_{high}	-17.1	11.8	-18.5	-0.2	-9.9	-3.7
M_{low}	-1.5	6.6	-16.9	-0.5	-3.7	-32.1

Table 2.33: Differences of the recoveries of each laboratory and spiking level compared to the mean recovery. Inf = infant formula, Ani = animal feeding stuff, Pea = peanut butter, Pap = paprika powser, Pis = pistachio paste, Fig = fig paste. The used laboratory codes was taken from the first collaborative trial on various foods

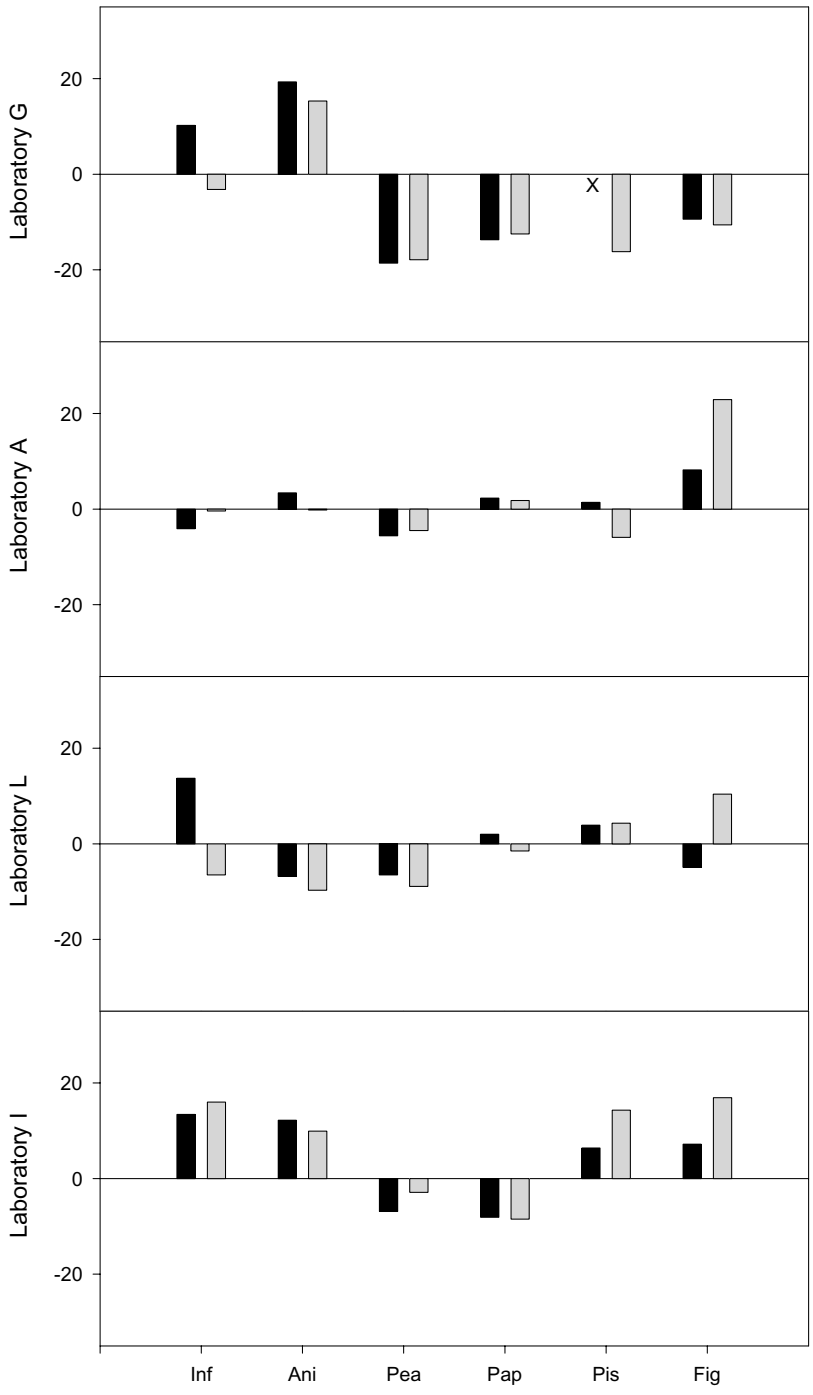


Figure 2.35: Recovery deviation from the mean recovery [in %] for selected laboratories. Part 1. Black bars show higher spiking levels. Outlier values are marked with an X

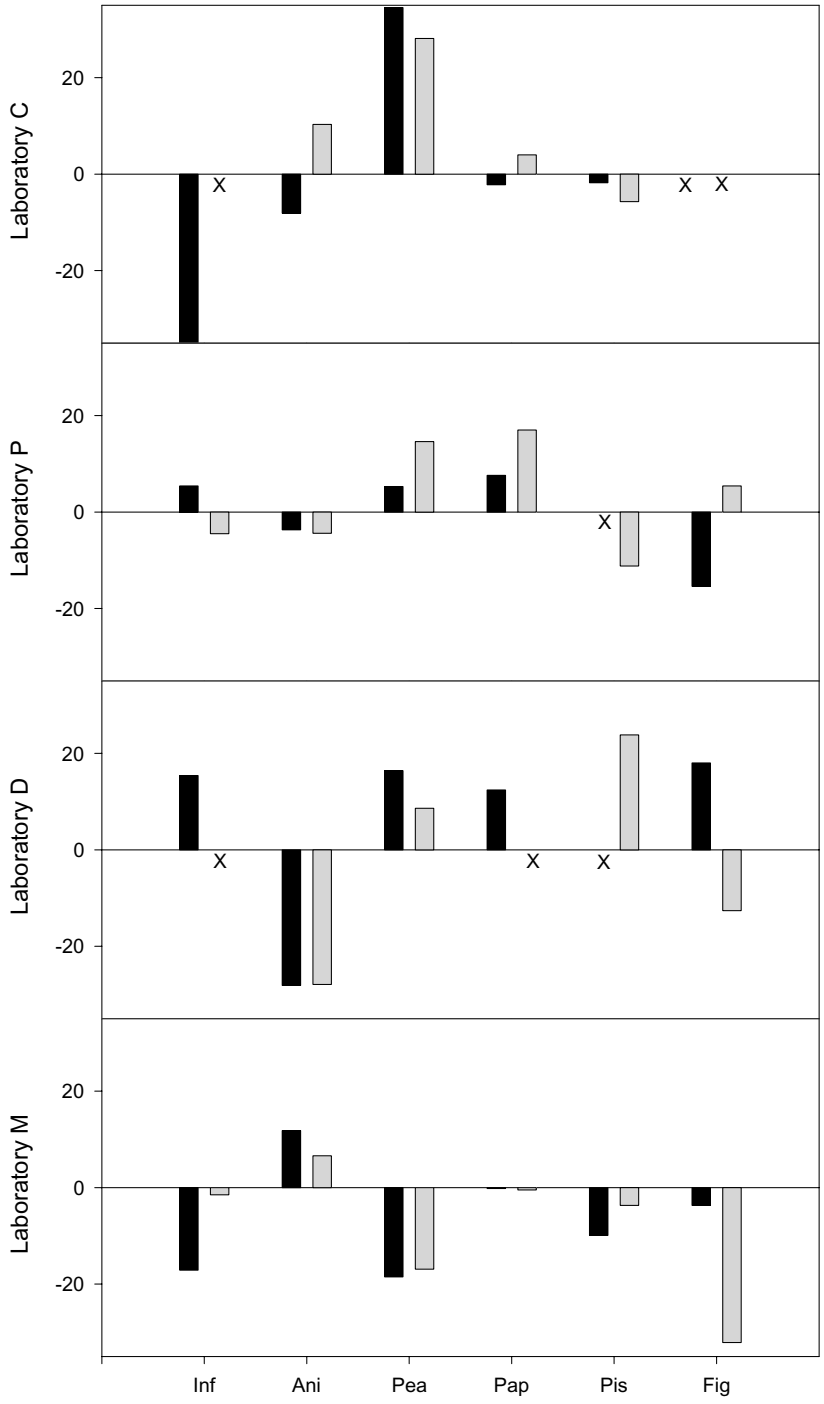


Figure 2.36: Recovery deviation from the mean recovery [in %] for selected laboratories. Part 2. Black bars show higher spiking levels. Outlier values are marked with an X

reported several recoveries with less than 5% deviation (A and L) and did not exceed more than 15% deviation for the rest of the reported recoveries[19]. These observations were a clear indicator for a laboratory dependent method precision, that seemed to be independent from the method itself.

To unveil any correlation between the above described precision (»*matching*« recoveries) and the overall deviation for a laboratory, the average value of all differences (Δ_{hl}/n) from the corresponding recoveries:

$$(\Delta_{hl} = \sum_{i=1}^{n} |Rec_{high} - Rec_{low}|_n)^{20}$$

was compared with the average difference for all recoveries (Δ_{abs}/n) for a single laboratory:

$$(\Delta_{abs} = \sum_{i=1}^{n} |Rec_n - Rec_{mean}|)^{21}$$

. In the case of single outliers the (corresponding) remaining recovery value was ignored for the determination of Δ_{hl}, while this value was taken into account for the determination of Δ_{abs}, since these values were valid results (Table 2.34. When the calculated precision parameters for each laboratory are plotted against each other (Figure 2.37), it can be seen, that no correlation can be made between the »*in-house*« precision for a laboratory and its precision of the recovery value compared to the mean recovery (of all laboratories).

Laboratory code	$\sum_{i=1}^{n} \lvert Rec_{high} - Rec_{low}\rvert_n$			$\sum_{i=1}^{n} \lvert Rec_n - Rec_{mean}\rvert$		
	n	Δ_{hl}	Δ_{hl}/n	n	Δ_{abs}	Δ_{abs}/n
G	5	20.6	4.1	11	147.0	13.4
A	6	30.8	5.1	12	60.7	5.1
L	6	44.6	7.4	12	79.0	6.6
I	6	26.8	4.5	12	122.8	10.2
C	4	35.0	8.8	9	131.7	14.6
P	5	50.2	10.0	11	94.5	8.6
D	3	38.7	12.9	9	163.3	18.1
M	6	57.3	9.6	12	122.3	10.2

Table 2.34: Relative precision indices for the different laboratories

[19]One exception was the '*low*' recovery of laboratory A for fig paste

[20]Index for the "within"laboratory precision

[21]Index for the precision for a laboratory compared to mean value of all laboratories

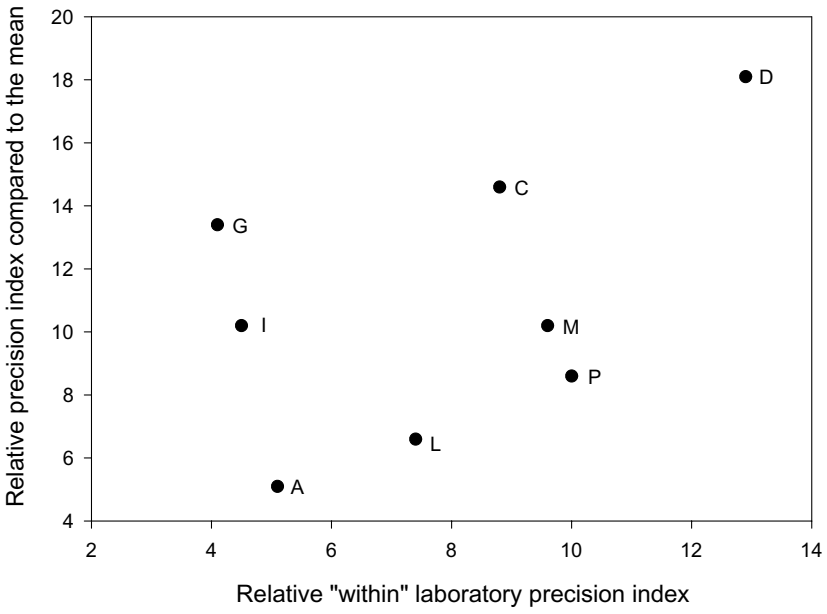


Figure 2.37: Correlation of the calculated precision parameters from Table 2.34

To summarize, it can be said that no trend in the results from matrix to matrix was observed for any laboratory, while for the discussed precision data no correlation was found within the selected group of laboratories. However, it was revealed that differences in the precision between laboratories existed, and that the number of outliers was found to be an indicator for the performance of the remaining valid data within the laboratory group.

2.4 Development of Simplified Densitometers

2.4.1 Background

Several methods for the determination of aflatoxins after TLC-separation are available and commonly used depending on the availability of technical equipment. The far most easiest method is the previously described visual judgment under UV-light (Chapter 2.2.1). This technique requires a mercury lamp with an emission of 366 nm[22]. However, results strongly depend on the visual skills of the analyst. Another drawback of this detection approach is that no documentation such as a densitogram or photograph is available to support the results if needed later on.

The alternative to visual judgment of spot intensities is the densitometric determination of the aflatoxin spots on the TLC-plate. Densitometry allows to measure the fluorescence of aflatoxins under UV-light in transmission or reflection mode. Modern aflatoxin densitometry uses exclusively fluorescence reflection for measurement, since the transmission mode requires transparent TLC-plates.

Nowadays, commercially available fluorescence densitometers have been developed to state-of-the-art products with excellent performance characteristics concerning precision, data resolution and flexibility. However such instruments are rather bulky (due to the broad applicability for most TLC applications) and depend on connection to the mains. Therefore a new device has been developed with the intention to deliver a simplified, inexpensive and precise alternative to commercially available TLC densitometers.

The main approach was to substitute the currently used components such as mercury gas tubes and photomultiplier with state-of-the-art semiconductors. The most recent availability of light emitting diodes (LED) with a peak wavelengh of 370 nm initiated the first considerations of a miniaturised low-power densitometer. In the past several approaches have already been made to elaborate and validate means for the fluorescence determination on TLC plates with commercially available or simple prototype apparatus [147–154],

[22]Maximum excitation wavelenght for aflatoxins

while some more recent approaches considered high tech laser applications for the determination of aflatoxins on TLC plates [155, 156].

However, lasers are still fairly expensive instruments [157] although available in small, which makes them undesirable for the goals described here. Impressive results have been achieved by simple means with so-called »*spotmeter*« prototypes [147, 148] that even used simple photoresistors and mercury lamps for fluorescence measurement. These devices were simple in its construction and dedicated to determine the fluorescence with a probe that was positioned over the aflatoxin spot. Spots as low as 1 ng were recorded. However all '*spotmeters*' measured the fluorescence by transmission on the TLC-plate, which was induced by mercury gas tubes.

More recently, with the availability of modern photographic and computer equipment, the use of digital cameras or common office scanners has been described for the use of TLC measurements [105, 158, 159].

2.4.2 General Requirements for Aflatoxin Densitometers

One of the most important requirements for any aflatoxin densitometer alternative is the applicability in view of current legislative limits. Therefore any device must have a sufficiently low limit of detection (LOD) and limit of quantification (LOQ) in combination with an appropriate TLC method). Furthermore the device must deliver reproducible and stable signals, thus no significant drift or fade of the signal[23] shall influence the measurement. Additional features such as simplicity, low power consumption or low production costs were highly desired for these alternatives.

2.4.3 Development of a Semiconductor based Densitometer Cell

The implementation of the novel UV-LED (peak emission at 370 [nm]) was an exceptional approach, since no other commonly available alternative to mercury lamps exist for the excitation of aflatoxins at the desired wavelength (Figure 2.38[24]).

In addition, the substitution of the photomultiplier usually utilized was necessary in order to miniaturise and reduce the power consumption of the device. The only appropriate sensor was found to be a novel photo-diode

[23] e.g. degradation of the aflatoxins during the measurement

[24] Reprint with permission of Alex Ryer, International Light

with a maximum sensitivity at 440 [nm]. As can be seen in Figure 2.39[25] depending on the type of photodiode, the sensitivity is about 100 to 100.000 time less than those of photomultipliers [160]. The photo diode used here was based on **Ga**llium **P**hosphorus (GaP) and had a typical sensitivity of 0.16 [A×cm^2/W] at 440 [nm] which was significantly inferior to photomultipliers.

In the early stage of this study a pre-evaluation test was performed on a flexible work bench (Figure 2.40) to generally determine the possibilities of the detector and to obtain indications if the system is sensitive enough for the desired application.

Thus, the UV-LED (370 [nm] peak emission), a cut-off filter (418 [nm] short-pass) and the photodiode (440 [nm] peak sensitivity) were mounted on a support made from cardboard paper for first experiments (Figure 2.41). The schematic draw of this device is given in Figure 2.42 and was kept during all experiments.

In addition, a power supply module was used, which delivered 12 V DC. For signal amplification a previously published electronic circuit was modified and fit to implement the LED and photo diode. The circuit design was based on an application dedicated for the measurement of illumination [161] and is based on generic electronic components. The layout of this circuit is given in Figure 2.43.

The fluorescence of the aflatoxin was excited with the UV-LED and

[25]Reprint with permission of Alex Ryer, International Light

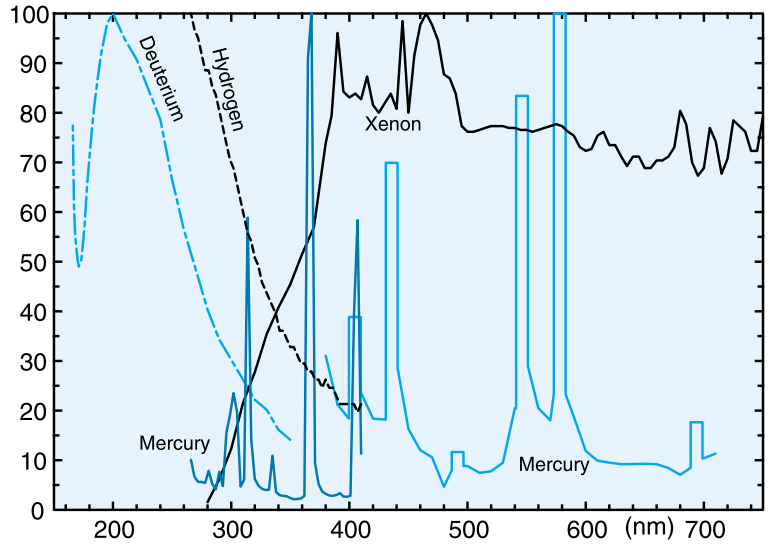


Figure 2.38: Relative emission spectra of different light sources

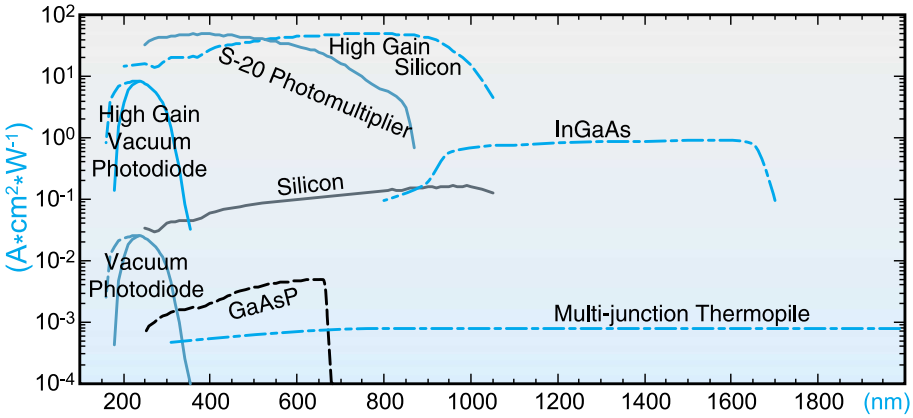


Figure 2.39: Sensitivity of different photo sensor types

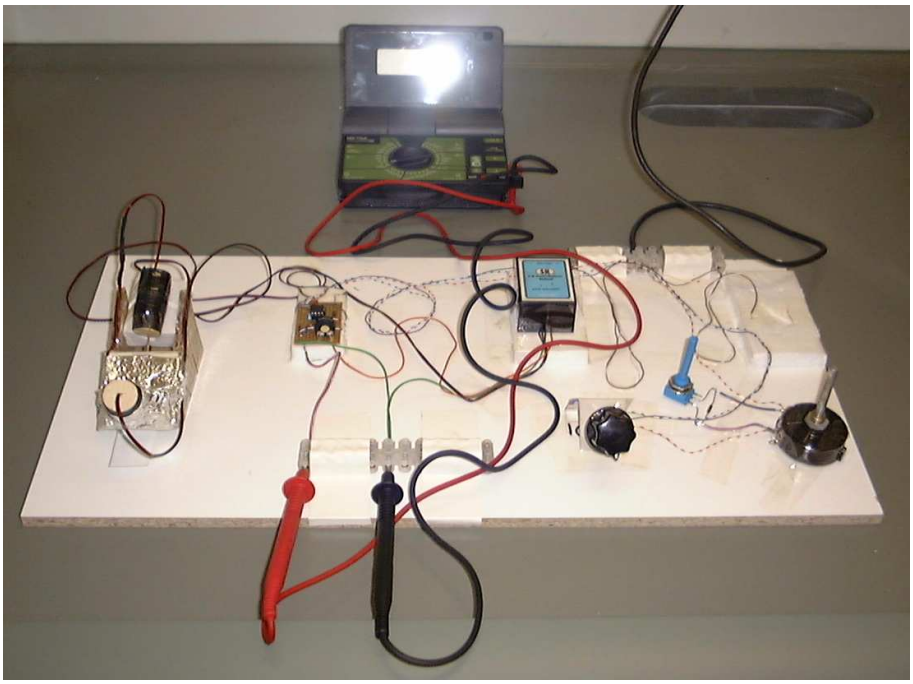


Figure 2.40: View on the early stage of the testing workbench

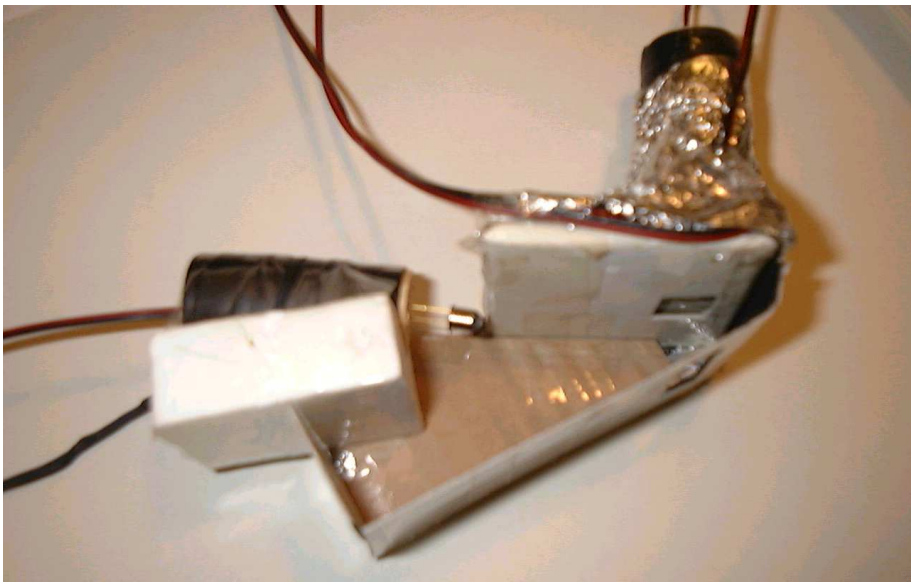


Figure 2.41: View on an early prototype of the SeBaDeC

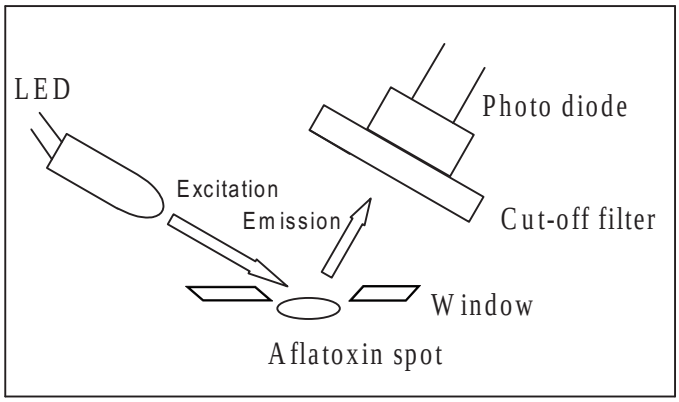


Figure 2.42: Schematic draw of the SeBaDeC

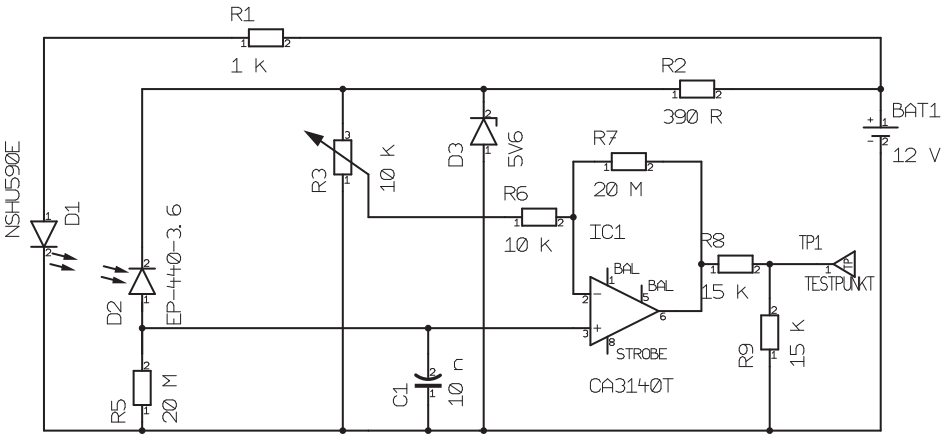


Figure 2.43: Layout of the first prototype circuitry

was measured after the cut-off of the excitation light using a cut-off filter ($\geq$418 nm). The fluorescence was then transformed into an electrical signal by the photodiode, this signal was amplified and directly measured with a digital multimeter. Generally the circuit functioned as such:

The LED (D1) is put in-line with an resistor (R1) to limit the current to 10 mA. The UV-light that is emitted by D1 induces the desired fluorescence on the aflatoxin spots. This fluorescence (light with a longer wavelength than the excitation wavelength) is separated with the cut-off filter and measured by the photodiode (D2).

This photodiode operates in such a way that in the case of absence of light (no photons entering the semiconductor layer) the resistance is high, allowing a current of approximately 8 pA to flow [162]. Due to the sensitivity of D2 to light at 440 nm, any fluorescence results in an electrical current (within D2) that is linear to the amount of fluorescence [163]. This results again in a linear change of voltage at the junction of D2 and R5 [164] and is amplified by the operational amplifier (IC1). IC1 amplifies the voltage difference between the pin 2 and pin 3 of IC1. The pins 1, 5 and 8 are of no interest in this case, while the voltage on pin 2 of IC1 is adjusted with the potentiometers R3 and R4. Two potentiometers were used with different values for coarse and fine adjustment. The ratio of R7/R6 determined the amplification factor which

was 2000 in this example. On pin 6 the amplified signal was divided by the resistors R8 and R9 and directly displayed on an attached digital multimeter (DMM).

First experiments with this construction (Figure 2.41) indicated that amounts of 5 ng of aflatoxin B_1 yielded in definite signals on the DMM. This gave a strong indication that with further improvements of the detector cell design and the electronic circuits, it should be possible to detect smaller amounts. Furthermore, the data of the preliminary experiments indicated that measurements were highly influenced by smallest amounts of scattering light in a dark room, as well as the natural electrical field of the operator's hand. Hence any movement of the operator influenced the signal. To eliminate these unwanted effects a fully light and electrical field shielded detector cell was considered be crucial for further experiments. Therefore such a discrete metal container (hosting the UV-LED, the photodiode and the cut-off filter) was made. In addition, the wiring from the detector cell to the amplifier was made with shielded cables in addition. In order to allow a reliable and easy data processing, the amplified signal was directly converted into digital data with a simple analogue-digital-converter (ADC) in combination with an easily understandable software [165]. The origin of this hardware-software combination (ADC) was subject to a submission for a contest in 1998 and thus was fully described. The author of the software approved the free use without any restrictions. This set-up allowed direct data recording to the parallel port of a PC. The circuit layout of this preliminary version is shown in Figure 2.44 and functions as follows:

The LED D1 was operated in series with R1 and powered by a constant voltage controlled by IC9. IC9 is a variable voltage controller and can be adjusted by the potentiometer R26. The *zener*-diode D10 and R2 function as a voltage stabiliser on the input segment of IC1.

The amplified signal was divided to fit to a range of 0 - 5 V by the potentiometer R23. This output signal of maximum 5 V was then converted into digital data by the IC3 (ADC). The diode pair D7 and D8 guaranteed that the voltage on the analogue input of IC3 (pin2) does not exceed 5 V during the calibration process to protect IC3. A more detailed description of the circuit can be found in [161, 165].

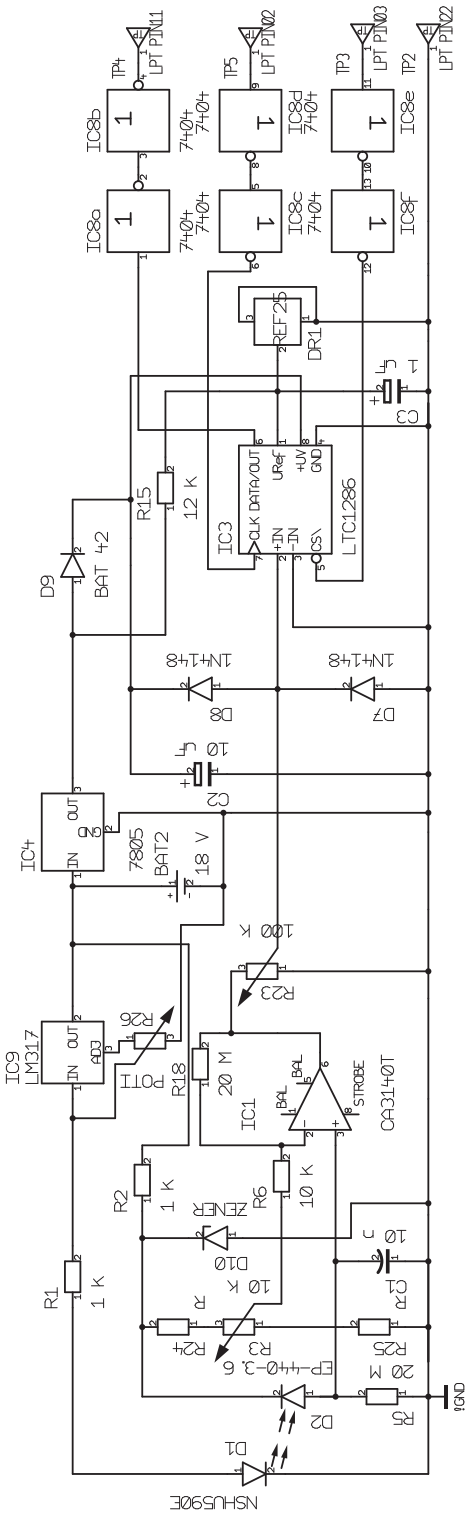


Figure 2.44: Layout of the final prototype circuitry

Generally, the way of signal recording and processing from the detector is not limited to the approach described here. Many kinds of data logging, data storage or processing systems are nowadays widely used in miniaturized devices of daily use (electronic thermometers, mobile phones). However, such approaches were not considered in this work, since they are subject to pure electronics. The actual aim of this work was the evaluation of the measuring principle with a miniaturized and simplified apparatus. The final prototype of the first model, is shown in Figure 2.45, while the latest model (SeBaDeC v0.2) is shown in Figure 2.46.

2.4.4 Characterization of the Densitometer Cell (Prototype version 0.1)

To sufficiently characterize the simplified densitometer cell and describe the limits of the discussed prototype, several parameters were elaborated and directly compared with results of an commercially available densitometer (CAS)[26].

Thin-layer chromatograms with aflatoxin B_1 concentrations ranging from 1 ng to 9 ng absolute per spot were developed and reflected contamination

[26]CAMAG TLC-Scanner3, CAMAG Switzerland

Figure 2.45: View of the SeBaDeC prototype version 0.1

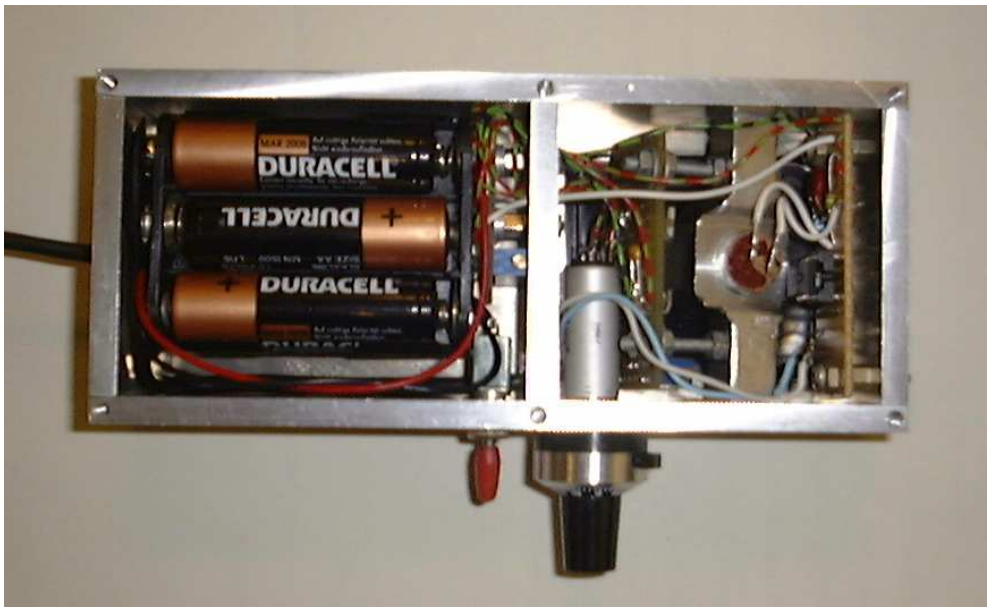


Figure 2.46: View of the SeBaDeC prototype version 0.2

levels of about 1 μg/kg and above[27]. Aflatoxin B_1 was chosen to be the single analyte for performance demonstration, since it is the predominant aflatoxin found in contaminated food products and is also explicitly regulated as a single contaminant [46]. Furthermore, this procedure drastically simplifies the measurement due to the fact that no other aflatoxin spots can interfere with the AfB_1 measurement.

For first evaluation of the detector cell, it was moved freely by hand along a ruler over the TLC-plate. The signal was recorded at a speed of 23[28] datapoints per second. Despite some fluctuations in the recorded data and a wobbling baseline that was due to the uneven movement of the detector by hand, an amount of 1 ng aflatoxin B_1 resulted in an identifiable signal in this preliminary experiment (Figure 2.47). The scan with the SeBaDeC was performed in an angle of 90° to the development of the TLC-plate in order to allow the simultaneous determination of all aflatoxin B_1 spots (spots with the same R_f-value) in one single scan.

A calibration curve was established after adjustment of the spot window (to fit with the size of the aflatoxin spot) and after improving the movement of the detector cell over the TLC-plate. The latter was achieved by support-

[27]These values were calculated assuming that the previously discussed TLC-method for aflatoxins is applied

[28]Sample-rate setting of the software

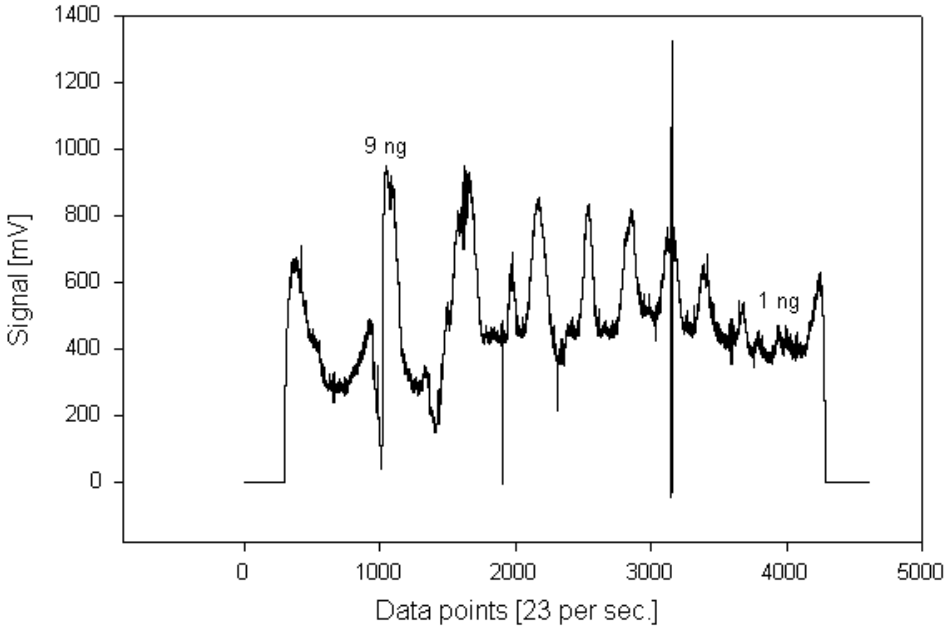


Figure 2.47: Densitogram with the prototype of the SeBaDeC

ing the movement of the densitometer cell by means of a simple threaded bold that pushed the detector along the plate when the bold was revolved manually. In addition the TLC-plate was mounted between two strips of TLC-plate material to allow a smooth movement of the detector cell at the edges of the plate. This setup successfully avoided the previously described fluctuations and wobbling. The right window size was found to be a crucial parameter, since a better signal was obtained after increasing the window and collecting the fluorescence of the whole spot.

The recorded data was read into Microsoft Excel97 ® and transferred into a diagram. This diagram was printed and the signals were measured in [cm]. For comparison, this TLC-plate was first scanned with the CAS and subsequently re-scanned with the SeBaDeC. Table 2.35 shows this data in addition to the densitometric results obtained with the commercially available scanner. The calibration curves are shown in Figure 2.48. This calibration procedure was repeated again for all four aflatoxins. Results are listed in Table 2.36.

These calibration experiments clearly show that the proposed simplification of the densitometer-cell by substitution of the commonly used components for densitometry (mercury lamp and photo multiplier) with state-of-the-art semiconductors (LED and photo diode) offers potential applications for densitometric measurements.

ng AfB_1	**CAS**	**SeBaDeC**
1	44.9	1.4
2	92.5	2.0
3	136	3.4
4	177.2	4.0
5	219.1	5.2
6	264.5	5.7
7	312.9	6.5
8	360.3	7.6
9	401.6	8.9
Correlation (r=)	0.9998	0.9961
LOD [ng]	0.4	1.5
LOQ [ng]	0.5	2.2

Table 2.35: Comparison of AfB_1 calibration results for the CAS and SeBaDeC

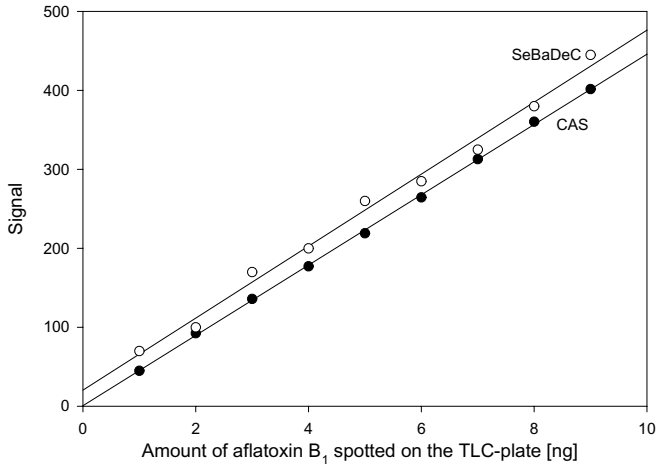


Figure 2.48: Comparison of calibration data from CAS and SeBaDeC

Analyte	**Af**B_1	**Af**B_2	**Af**G_1	**Af**G_2
Correlation (r)	0.9983	0.9954	0.9944	0.9504
LOD [ng]	1.2	1.7	1.7	4.8
LOQ [ng]	1.9	2.8	2.5	7.1
RSD [%] (method)	2.8	3.8	5.0	15.7

Table 2.36: Method performance of the SeBaDeC with aflatoxin standards

For further characterization of the detector, the long-term drift and the decay of the signal was investigated. Therefore the detector cell was positioned over an aflatoxin free spot of the TLC-plate and the signal was recorded for 50 minutes. The drift was found to be 1.8% over the measured time range (Figure 2.49). This indicates that during a scan time of approximately 3 to 5 minutes no measurable drift should occur.

As aflatoxins are subject to UV-light degradation [98], the radiation during the fluorescence measurement might effect results significantly. Signal fading rates of 50% within 3 min were reported with spotmeters [147] and limited the maximum radiation exposure of spots during measurement to 10 sec. However, the power ratings of the UV-LED are as low as 750 μW at a single small bandwidth of 370 nm. Contrary to this, previously described

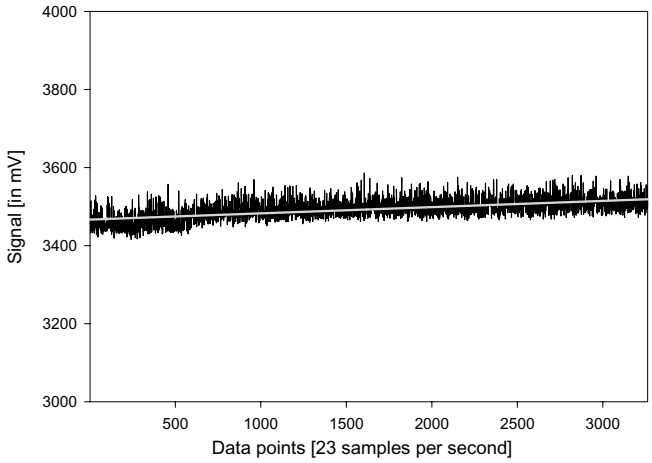


Figure 2.49: Stability of the SeBaDeC signal over a blank position

UV-light sources were based on fluorescent gas tubes or mercury tubes with significantly higher power ratings of several Watt. This led to the assumption that the described fade should be significantly lower for the SeBaDeC. For confirmation the detector cell was positioned over an aflatoxin B_1 spot (10 ng absolute) and the signal was recorded over a time range of 45 minutes and additionally for 10 minutes over a blank position (Figure 2.50). The signal fade was calculated to be less than 1.5% within 1 minute. This time was assumed to be the maximum exposure time for repeated measurements.

Finally, fortified test samples of paprika powder were analysed by TLC and the aflatoxin B_1 content was measured with both densitometers, the SeBaDeC and the CAS. Paprika powder was selected as test matrix, since it is known to be critical in terms of matrix interference (see Chapter 2.2.1). As shown in Table 2.37 the data obtained in this comparison are very similar which confirms that the proposed SeBaDeC is already capable to determine aflatoxin spots with a sufficient precision. However, more effort is foreseen in construction and electronics to achieve results which are comparable or even better than commercial densitometers.

Figure 2.51 shows the obtained chromatogram of the scan of aflatoxin B_1 standards and fortified paprika samples.

Therefore the SeBaDeC is a promising alternative in densitometry that offers new features such as the miniaturization of the densitomer-cell, low-

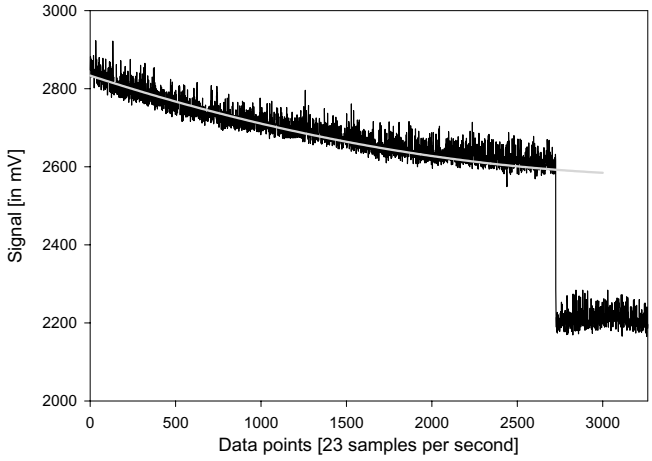


Figure 2.50: Decay of an aflatoxin B_1 signal when radiated with the SeBaDeC

Aflatoxin added [**in μg/kg**]	**AfB_1 found**		**AfG_2 found**	
	CAS	SeBaDeC	CAS	SeBaDeC
blank	0	0	0	0
1	0.9	0.9	0.9	0.9
2	1.5	1.6	1.5	1.7
3	2.8	2.5	2.7	3.0
4	3.3	3.0	3.0	3.3

Table 2.37: Calibration results for the SeBaDeC with aflatoxins B_1 and G_2

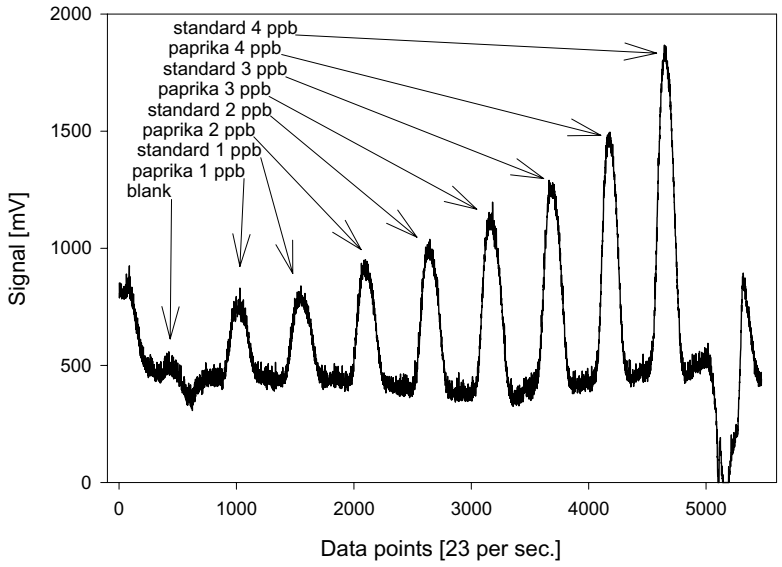


Figure 2.51: Densitogram of aflatoxin B_1 spots from paprika samples and corresponding standards

power requirements of less than 50 mA, a long life span and low costs of the components used. Further experiments were made with an improved cell design, while the electronic design remained mainly the same.

2.4.5 Improvements of the Densitometer Cell (Prototype version 0.1)

Since the feasibility of the proposed detector prototype version 0.1 has been shown successfully, further improvements were made by:

- implementation of the electronic circuits into the cell housing to shorten connections between components and thus reduce unwanted noise or interferences,
- shorten the pathway lengths of the optical system to increase the signal yield,
- changing the cut-off filter to a single band filter with 430 nm to improve the selectivity of the signal,
- removal of R2, R8 and D3 in the circuit (Figure 2.43) to increase the voltage drop,
- decreasing the amplification of the OA from 2000 to 65 (by changing R7 from 20 MΩ to 650kΩ to reduce the signal noise (Figure 2.43) and
- utilization of a commercial ADC (Gilson GISOC interface).

Measurements with the Prototype version 0.2

The effectiveness of this late stage prototype version 0.2 revealed further improvements in the measurements. Similarly to the previously described interference from static charges of the operator, it was found that the cell needed to be connected to ground (earth) for any measurements. The influence of grounding the housing for a blank signal is shown in Figure 2.52. Similarly to the previous test, a calibration series was made with AfB_1 spots in the range of 0.5 ng to 4.5 ng. Figure 2.53 shows such a densitogram, while in Figure 2.54 the corresponding calibration curve can be seen. The performance characteristics were again compared to the commercial scanner and are shown in Table 2.38.

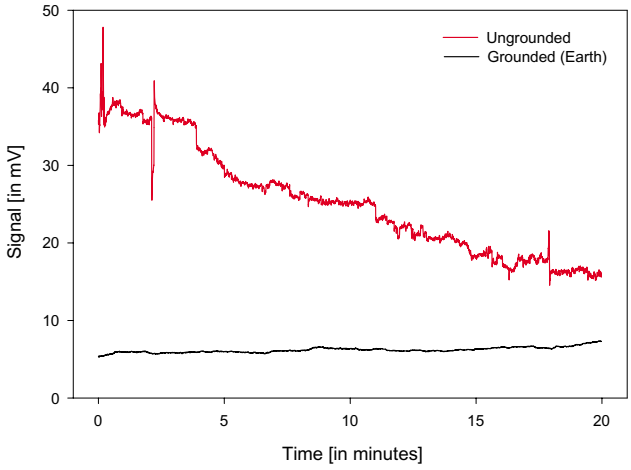


Figure 2.52: Blank densitogram obtained with and without the grounded housing

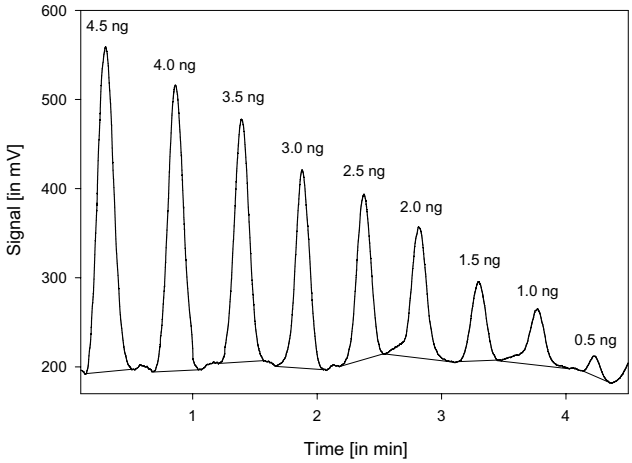


Figure 2.53: Densitogram of aflatoxin B_1 spots from 0.5 ng to 4.5 ng

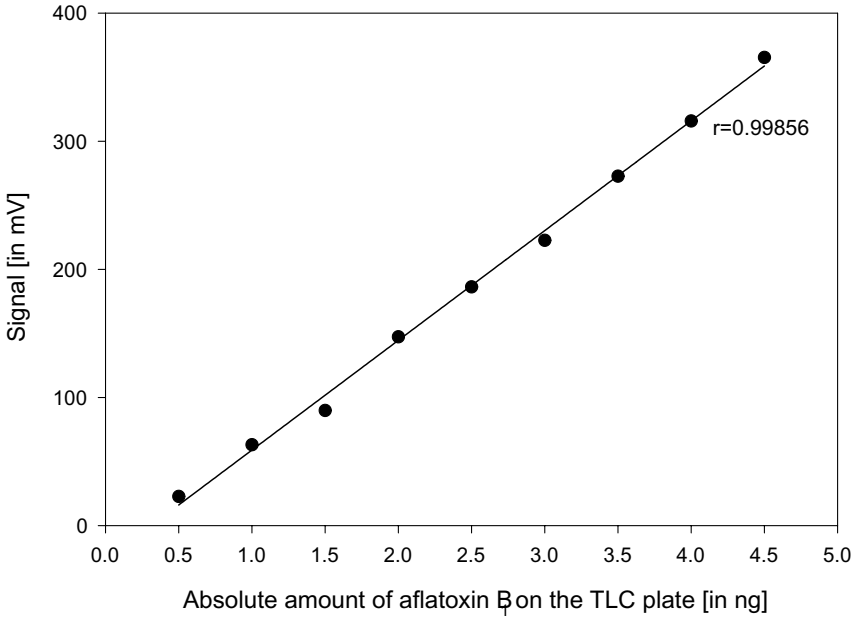


Figure 2.54: Calibration curve of aflatoxin B_1 (SeBaDeC v0.2)

As can be seen in Table 2.38 the performance characteristics of the SeBaDeC version 0.2 improved significantly. The performance obtained entered a range that can compare with the commercial scanner. However due to the time schedule of this work no further improvements were made thereafter.

SeBaDeC version 0.2			**Commercial Scanner**		
LOD [μg/kg]	LOQ [μg/kg]	[RSD %]	LOD [μg/kg]	LOQ [μg/kg]	[RSD %]
0.45	0.67	3.1	0.27	0.40	1.8

Table 2.38: Performance comparison of the SeBaDeC v0.2 versus the CAS for aflatoxin B_1

2.4.6 Modification of an Office Scanner

General Considerations

In addition to the above described SeBaDeC, another approach to deliver a simplified densitometer was the modification of a commercially available flat-board scanner to be used as densitometer. Commercially available flat-board scanners are widely used in combination with a personal computer (PC) and can be considered as standard components in office work. The wide availability of such devices led to the idea that they should be easy to obtain in most cases and might even be easily modified for the desired application.

Generally flat-board scanners contain a movable unit, housing the light source and a detector-module which are moved together at a close distance along the area which is to be scanned. Commonly used light sources are cold-cathode tubes, while charge coupled device-modules (CCD) are used as light detectors.

Such a CCD array detector provides the capability to acquire a full spectrum in the time it takes a scanning unit to sample a single wavelength. This detector exhibits a high quantum efficiency and a readout noise which is about 100 times lower than conventional photodiode array detectors. This high sensitivity provides the basis for a sensitive measurement and means that far less fluorescence (light) is necessary for accurate measurements, which in turn results in a lower LOD and LOQ.

However for the determination of fluorescence from aflatoxin spots a number of elements in the scanner had to be changed. First of all the light source had to be changed from the default tube to a UV-tube with a peak emission wavelength of 366 nm (mercury band) to a allow the excitation of aflatoxin fluorescence.

In addition the introduction of a cut-off filter system was considered to be implemented into the scanner system, which cut off the excitation light of 366 nm produced by the UV-light source. This filter should reveal a high contrast in terms of fluorescence and residue light derived by other causes.

Furthermore all UV-light sensitive parts of the scanner had to be protected sufficiently with a cover or by other means. To assure the success of this project, the construction and preliminary experiments were performed at the *Fraunhofer Institut für Optik und Feinmechanik* in Jena (Contractor), in close collaboration with myself. During the early stage of the project the minimum requirements and specifications were defined as follows:

- No special communication protocol was required, thus the software for obtaining the image data shall be the generic TWAIN-driver[29] provided by the scanner manifacturer.
- The image evaluation must be performed with a generic image evaluation software, which shall be independent from the modified scanner.
- The scanner must transfer the signal via a parallel-interface, thus allowing to operate it with most IBM compatible PCs.
- The device must achieve an optical resolution of 300 dpi[30] and a channel depth of at least 8-bit (256); preferably 12-bit (4096). This allows a sufficient resolution in the spot intensity,which is equivalent to a true colour mode of 36-bit (12-bit for each of the three colour channels (Red Green Blue)).
- A spot of 1 ng AflatoxinB_1 must result in a detectable signal.

Due to the large extent of the assessment for available office scanners, the contractor started to look for available material and suitable scanners for this project. Preliminary test were performed and strongly indicated that the foreseen project was technically possible.

Development of the Modified Scanner

Selection of the appropriate office scanner Commercially available office scanners share a similar mechanical construction with small deviations. The principle construction consists of a light-tube (VIS) with a diameter of 2 - 3 mm and a movable apparatus containing the CCD-module and the optical system. The light-tube is positioned close to the scanner glass-plate that supports the image during the scan.

A commonly available office scanner from UMAX (Model *Astra 2000 P*) was selected as a suitable candidate due to the following facts and features:

- Excellent notes in tests of appropriate computer journals.
- Available specifications of the used CCD-module[31].

[29]TWAIN stands for **T**echnology **W**ithout **A**n **I**nteresting **N**ame and defines the communication protocol between any image processing software and hardware devices (e.g. scanners) for the aquisation of image data.

[30]dpi = Dots per inch

[31]NEC μPD3798, see annex for further specifications

- The scanner is made out of non-fluorescent materials.

- The TWAIN-interface allowed a 36-bit colour depth of the scanned image.

- Parallel-port interface that required no special ports on a PC.

Selection of the UV-light source (UV-tube) Appropriate UV-tubes with an emission wavelength of 366 nm were evaluated for the replacement of the VIS-tube. Due to the dimension of the field (A4) to be scanned, as well as the dimension of the scanner itself, the tube should have a length of approximately 200 [mm] with maximal power ratings of 10 Watts, which is equivalent to the power ratings of normal scanners. The UV-tube *TL 6W/08 Blacklight Blue* from Philips was found to be appropriate and is a commonly used UV-tube in various fields, thus the availability of the tube should be guaranteed in the near future.

Despite the fact that this tube is designed to be run in a *hot cathode* mode, it can also be run in *cold cathode* mode. The power ratings of the tube are nominally 6 watts, which are equivalent to 0.75 watts of UV-A radiation. A drawback of this tube was the diameter of 16 mm, since the approximate diameter of tubes in scanner is 3 mm. However, another candidate as an appropriate tube was not identified.

After this selection, the homogeneity of the radiation along the tube axis was tested in dependence of the distance from the tube. As can be seen in Figure 2.55 the total radiation is fairly constant at several distances from 5.5 mm to 41.2 mm along the tube axis. The measured variations in the region of 5 cm to 15 cm along the tube axis were in the range of 10% around the mean, while the highest variation occurred at the ends of the tube.

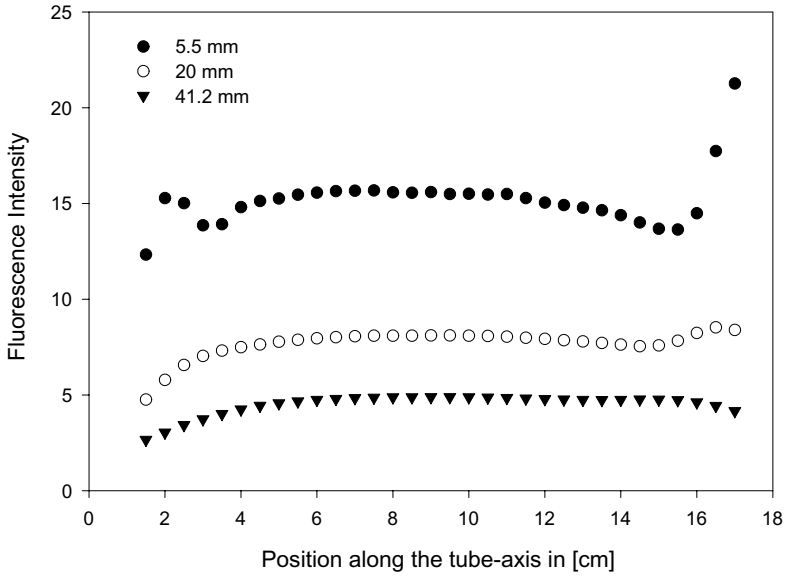


Figure 2.55: Radiation along the UV-tube axis at different distances

This effect can be explained due to the glowing of the cathods at the ends of the tube (see also Figure 2.62 concerning the red channel). However, if the measurement is performed in the mid range of the tube axis this effect should be of minor relevance. It was further assumed that it should be possible to compensate this effect with appropriate reflectors if needed, since equivalent solutions already exist in commercial scanners.

Selection of the appropriate cut-off filter Several cut-off filters with various defined cut-off wavelengths are commercially available. These filters must allow the penetration of the fluorescence, while they have to block the light emitted by the UV-tube (excitation light). In addition the filters must have an insignificant self-fluorescence. Filters with these properties were purchased and tested. Since the excitation of the fluorescence was at 366 nm and the emission was 420 nm and above, the filters must have a cut-off wavelenght of 370 mn to 420 nm.

Three filter types, KV-399, KV-408 and KV-418[32], were tested for their

[32]KV is an abbreviation for **K**unststoff**V**erbund-Filter; the number indicates the wave-

suitability to obtain large fluorescence signal. These filters were recommended by the supplier, since the KV-filter series is characterized by a low self-fluorescence of the filter material[33].

For evaluation of the suitability of the filters as well as the UV-tube intensity, a previously developed TLC-plate with aflatoxin B_1, B_2, G_1 and G_2 spots in the range of 1 - 4 ng was radiated and the fluorescence was recorded with a CCD-camera, while using the various cut-off filters to suppress the excitation light.

All three filters (KV-418, KV-408 and KV-399) resulted in fluorescence signals. However the best contrast of spots from the background was obtained with the KV-418 filter (Figures 2.56 to 2.58.

These signals (RGB[34]-signals from the TLC-plate) were separated in its single channels of blue, green and red and were further processed[35]. As can be seen, the blue channel revealed the highest contrast against the background (Figure 2.59), while the green channel signals were much lower for all aflatoxins, including AfG_1 and AfG_2. (Figure 2.60).

The red channel resulted in no signals at all. Both figures of the blue and green channel data were obtained from right set of four spots equivalent to approximately 1 ng each aflatoxin on the plate. Even though the distance of the UV-tube was 10 cm (mean distance) from the TLC-plate and the distance

length [in nm] at which a 50% transmission is achieved.

[33]Personal communication with the a technician from SCHOTT-Mainz

[34]Red Green Blue

[35]Mathematica ® 4.0

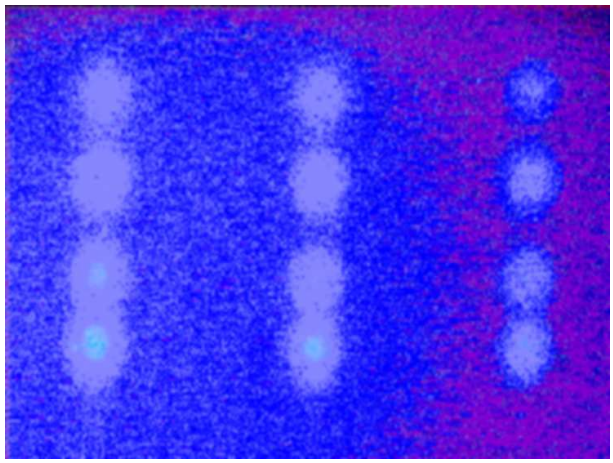


Figure 2.56: Contrast of fluorescence with the filter KV-399

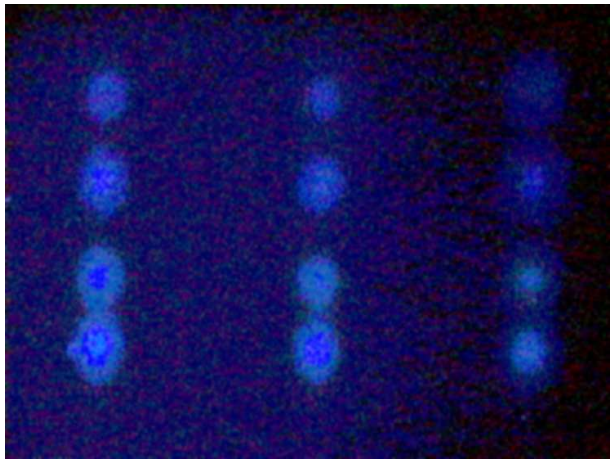


Figure 2.57: Contrast of fluorescence with the filter KV-408

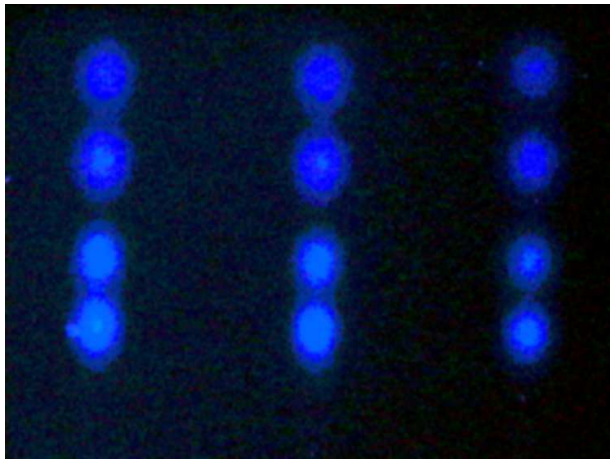


Figure 2.58: Contrast of fluorescence with the filter KV-418

of the CCD-Camera was 32 cm (mean distance) to the plate, the signal was intense at an exposure time of 1/50 second.

These results gave strong indications that the foreseen design is feasible, since the distance between the TLC-plate and the UV-tube as well as the TLC-plate and the CCD-module of the office scanner will be much smaller, even though the estimated exposure time will be at around 1/200 second during the scan.

After these preliminary experiments the modification of the scanner was started. Briefly the modifications were the following: Elevation of the glass plate (image support), in order to allow the implementation of the UV-tube (with a larger diameter). The cut-off filter (KV-418) was positioned in front of the CCD-module, and the optical systems finally adjusted. After mounting all parts, additional tests were performed in order to characterize and validate the modified scanner.

Characterization of the Modified Scanner

Prior to any aflatoxin measurements, general tests to determine the usable area for measurements and the linearity of the fluorescence intensity according to the position were performed. As previously shown in Figure 2.55 the radiation of the UV-tube depends on the position along the tube axis,

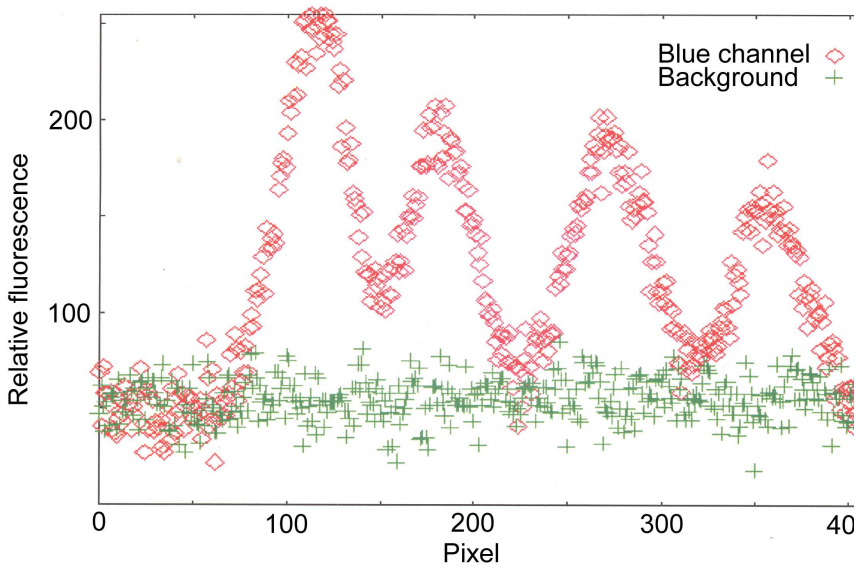


Figure 2.59: Fluorescence in the blue channel

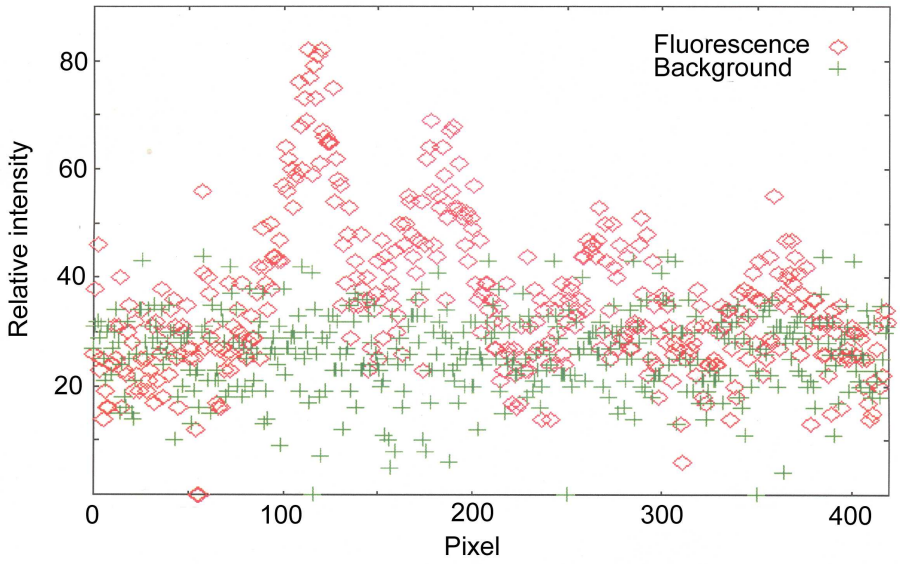


Figure 2.60: Fluorescence in the green channel

while in addition the dimension of the UV-tube employed were different from those of the original tube. Thus, the fluorescence of a spot is likely to differ depending on its position.

For testing purposes a fluorescent filter (GG17)[36] which emits fluorescence in a wide range of the green spectra was positioned on the image support. The resulting relative fluorescence is shown in Figure 2.61. As can be seen, in a range of 40 mm (left side) to 165 mm (Δ=125 mm) a suitable fluorescence can be obtained for the blue channel.

[36]Schott Mainz

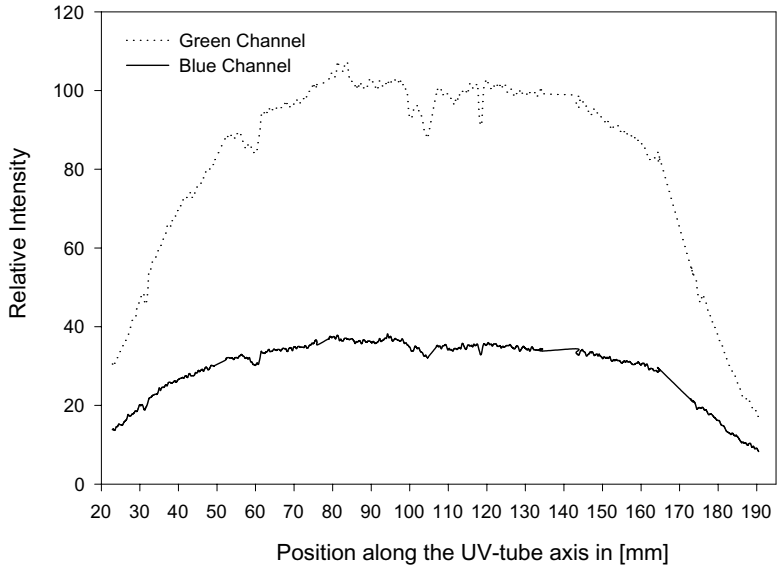


Figure 2.61: Relative fluorescence in the blue- and green-channel along the UV-tube

The observed fluorescence variation was ±10% (peak to valley) in the selected range. These observed variations were found to be reproducible and are thought to be due to differences in the coating of the tube. However, since it is foreseen to perform the calibration of unknown aflatoxin spots at the same location (position along the UV-tube), these variations are neglectable for further determinations.

In addition to this test, the influence of the cathode glowing was determined, since this effect can be seen on scanned images as bright red stripes along the image and contributes most to the background signal. Figure 2.62 shows this effect, which almost exclusively effects the red-channel, while the blue-channel is significantly less affected[37].

[37]This is important for the measurement of fluorescence at about 440 nm.

Figure 2.62: Effect of the Cathode Glowing on the different CCD-Channels

After these characterizations TLC-plates with different levels of aflatoxins were measured.

Results of TLC-plate Measurements

To measure the signal intensities of aflatoxin spots on the TLC-plate, a series of aflatoxins in the range of 1 ng to 9 ng for AfB_2 and AfG_2, respectively 0.5 ng to 4.5 ng for AfB_1 and AfG_1 were spotted and developed. The resulting chromatogram was first scanned with the CAS and subsequently re-scanned with the modified scanner. Figure 2.63 shows the obtained image (AfB_1, AfB_2, AfG_1 and AfG_2 from top to the bottom), while Figures 2.64 to 2.67 show the corresponding spot intensities as graphical plots.

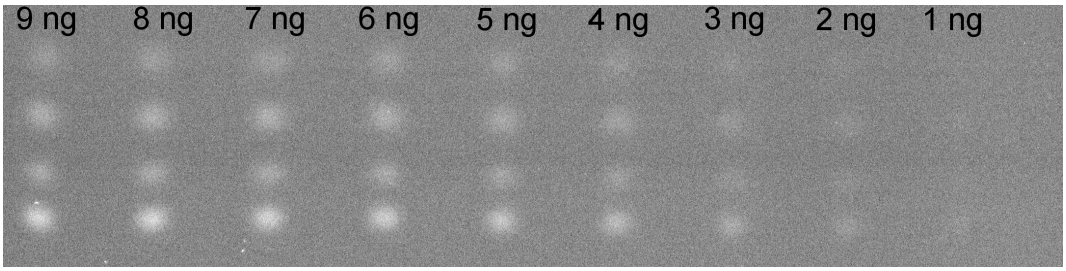


Figure 2.63: Image (8-bit blue channel only) of a TLC-plate scan

It was shown that spots of 1 ng each aflatoxin (representing a contamination level of ≈1 μg/kg) result in distinct signals. Table 2.39 summarizes the obtained parameters (LOD and LOQ) for the modified scanner. These values were derived from the 95% confidence interval of the corresponding calibration curves. The results were found to be satisfactory and show that the device is adequate for the determination of aflatoxins at the discussed level of ≥1 μg/kg.

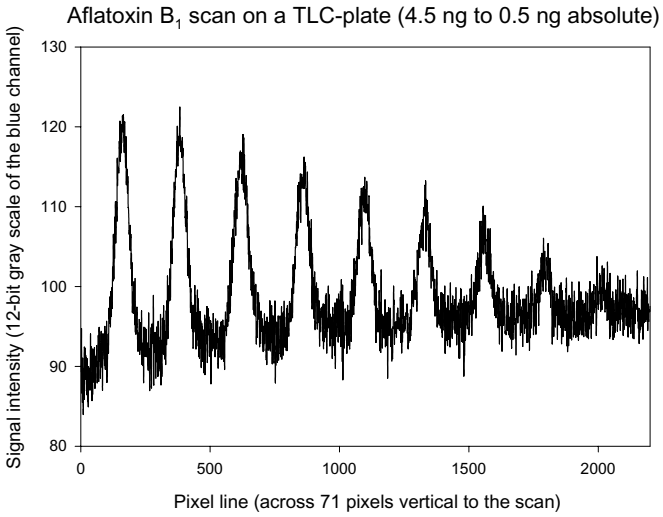


Figure 2.64: Spot intensities of the blue channel image (AfB_1)

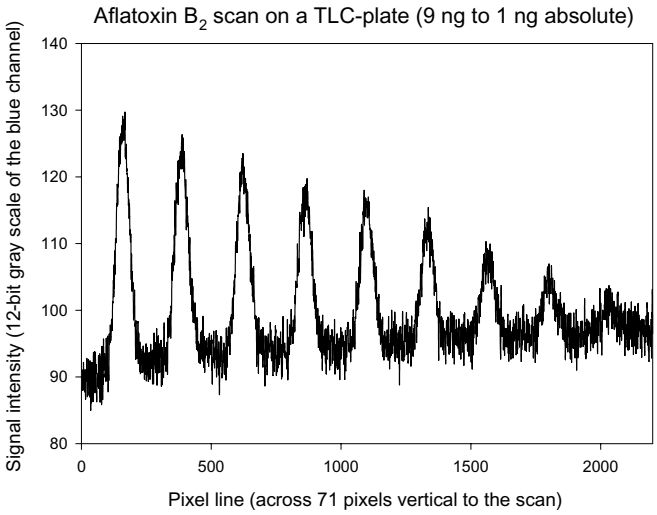


Figure 2.65: Spot intensities of the blue channel image (AfB_2)

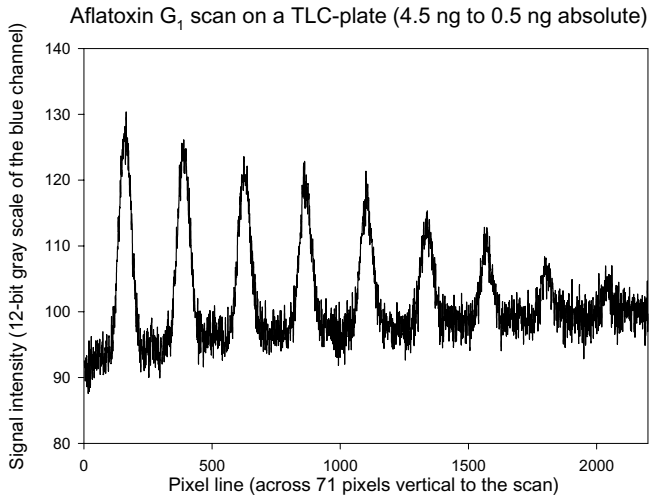


Figure 2.66: Spot intensities of the blue channel image (AfG_1)

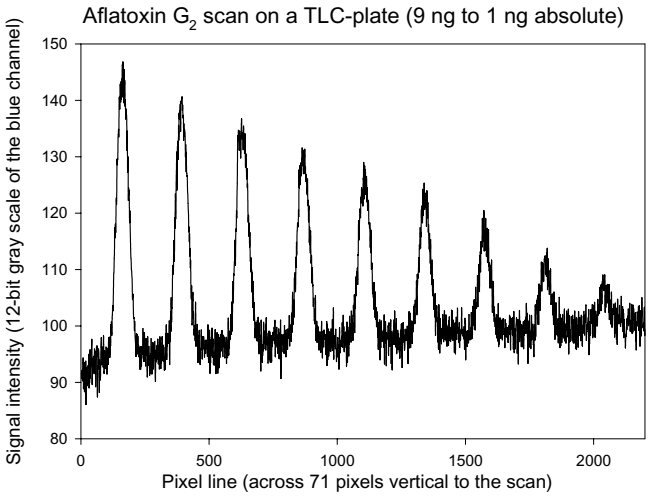


Figure 2.67: Spot intensities of the blue channel image (AfG_2)

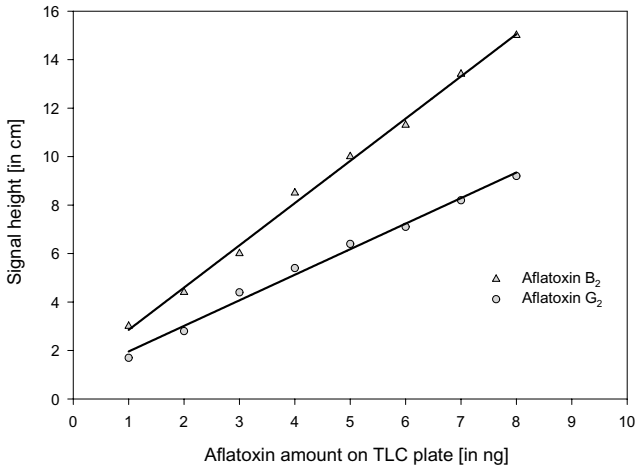


Figure 2.68: Calibration curves for Aflatoxin B_2 and Aflatoxin G_2 (modified scanner)

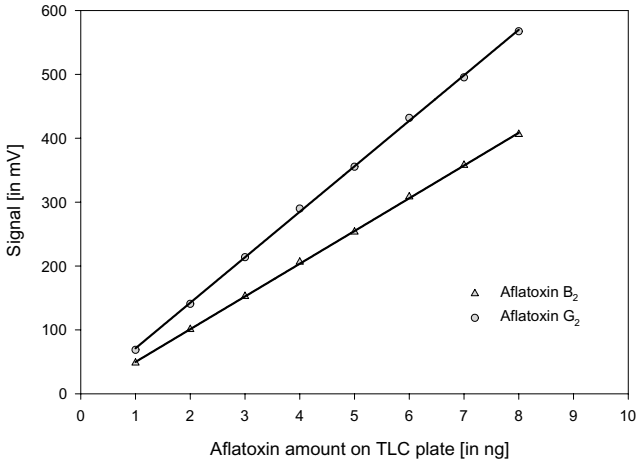


Figure 2.69: Calibration curves for Aflatoxin B_2 and Aflatoxin G_2 (CAS)

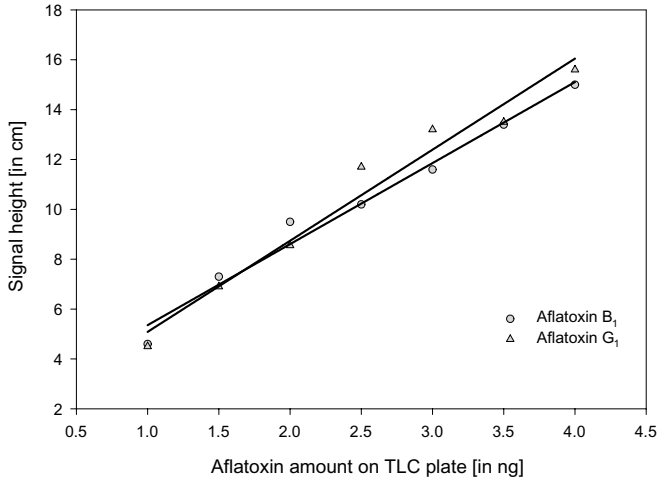


Figure 2.70: Calibration curves for Aflatoxin B_1 and Aflatoxin G_1 (modified scanner)

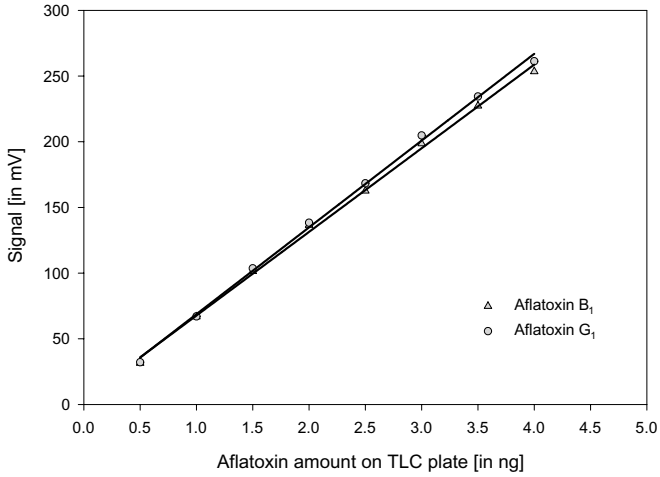


Figure 2.71: Calibration curves for Aflatoxin B_1 and Aflatoxin G_1 (CAS)

Analyte	**CAS**			**Modified Scanner**		
	LOD [μg/kg]	LOQ [μg/kg]	RSD [in %]	LOD [μg/kg]	LOQ [μg/kg]	RSD [in %]
AfB_1	0.4	0.6	2.7	1.3	1.9	7.6
AfB_2	0.2	0.4	0.9	0.9	1.4	3.2
AfG_1	0.3	0.5	2.5	1.2	1.7	7.1
AfG_2	0.6	0.8	2.0	1.4	2.0	4.9

Table 2.39: Results of the comparison CAS vs modified scanner

Chapter 3

Experimental

3.1 Materials

1. Reagents: All reagents were of recognized analytical grade. Unless stated differently, the water used complied with grade 3 of ISO 3696

   (a) Sodium chloride - Merck, Darmstadt [Germany]

   (b) Phosphate buffered saline (PBS) pH 7.4 - Sigma Aldrich, Milano [Italy]

   (c) Pyridinium hydrobromide perbromide (PBPB) [CAS: 39416-48-3] - Sigma Aldrich, Milano [Italy]

   (d) Potassium bromide - Merck, Darmstadt [Germany]

   (e) LC grade acetonitrile - Merck, Darmstadt [Germany]

   (f) LC grade methanol - Merck, Darmstadt [Germany]

   (g) Methanol (pure) - Merck, Darmstadt [Germany]

   (h) LC grade water, complying with grade 1 of ISO 3696

   (i) Extraction solvent: methanol - water solution [8 + 2 (v/v)]

   (j) n-Hexane (pure), cyclohexane (pure) or petrolether 60-140 - Merck, Darmstadt [Germany]

   (k) Nitric acid - Merck, Darmstadt [Germany]

   (l) LC mobile phase solvent (A) Water/acetonitrile/methanol solution [6 + 2 + 3 (v/v/v)]

   (m) LC mobile phase solvent (B) For use with electrochemically generated bromine: Water/acetonitrile/methanol solution [6 + 2 + 3 (v/v/v)] containing 120 mg potassium bromide and 350 mL nitric acid at 4 mol/L

(n) Post column reagent Dissolve 25 mg PBPB in 500 mL H2O. Solution can be used for up to four days if stored in a dark place at room temperature. This solution was only used in combination with LC mobile phase solvent (B)

(o) Toluene - Merck, Darmstadt [Germany]

(p) Aflatoxins in crystal form - Sigma Aldrich, Milano [Italy]

(q) Sucrose - Merck, Darmstadt [Germany]

(r) Paraffin [liquid] - Merck, Darmstadt [Germany]

2. Apparatus:

(a) High-performance Liquid Chromatograph:

i. Gilson HPLC pump system; pump models: »306« and »307«

ii. Gilson *Unipoint* software, version: 1.71

iii. Gilson ASPEC system; Automated Sample Preparation, Extraction and Collection device; model: »ASPEC XL«

iv. Supelco LC-18 HPLC column, 25 [cm] - 4.6 [mm] i.d.

v. Phone-Diagnostics KOBRA-cell®

vi. Waters fluorescence detector; model: 474

vii. Gilson UV-VIS detector; model: »V119 «

(b) Water Determination

i. Metrohm, Karl-Fischer water titration unit; model: »719 S Titrino «

(c) Thin-layer Chromatography

i. CAMAG sample application device; model: »Linomat«

ii. CAMAG *Cats* software, DOS-version

iii. CAMAG TLC-scanner; model: Scanner3

3.2 Methods

3.2.1 Sample Extraction

Generally a test portion of 50 g was extracted with a mixture of methanol-water (8 + 2 [v/v]). Depending on the extracted food matrix specific modifications had to be made. For the extraction of fig paste, peanut butter and pistachio paste the material was extracted for 3 minutes with a high speed blender. Fatty commodities (peanut butter and pistachios) required

the addition of 50 mL of n-hexane (or cyclohexane or petrolether) to the extractant to form an emulsion to allow a sufficient extraction. Paprika powder was extracted by shaking for 30 minutes (provided that the powder is ground sufficiently). This allows to process several samples simultaneously and reduces the risk of cross contamination. After extraction the extractant was subsequently filtered and diluted with PBS.

3.2.2 Immunoaffinity Clean-up

The filtered sample extract was diluted with water or PBS to a concentration of less than 11% MeOH and directly applied on the IAC (HPLC procedure) or filtered through a micro-fibre filter (TLC procedure). The diluted filtrate which had to be equivalent to 2.5 g of sample material was passed through the IAC at a flow rate of maximum 5 mL/min. The column was washed with water (HPLC procedure) or the column was washed with a washing solution containing Tween-20 and 11 % of methanol followed by water (TLC procdure). The IAC was gently dried by applying a light vacuum for 3 to 5 s.
The aflatoxins were eluted in a two step procedure. First a volume of 0.50 mL methanol was passed on the IAC. After 1 minute a second portion of methanol [1.0 mL] was passed on the IAC. The purified aflatoxins were collected in a volumetric flask and diluted with water to a defined volume (HPLC procdure) or they were collected in a glass vial (acid washed) which contained 50 μL of an MeOH-formic acid solution. This solution was subsequently dried under a stream of nitrogen (TLC procdure).

3.2.3 Re-dissolving for TLC Application

The dried aflatoxins were re-dissolved in 150 μL of a hexane-acetone-methanol solution (90 + 5 + 5 [v/v/v]). This procedure was performed in a sealed 2 mL glass vial.

3.2.4 HPLC Procedures

Injection of the Aflatoxins into the HPLC-system

The solution with the purified aflatoxins was injected into a HPLC injection port. The injection by total loop mode guarantees maximum accuracy. The total loop mode was carried out according to the injection port manufacturer[1]. Therefore a sample volume of 3 times the injection loop size was used

[1]Rheodyne

and at least 2/3 of this volume was injected into the valve, to ensure that the middle fraction remains in the injection loop. Thus, the loop was rinsed with the injection solvent while enough solvent remained in the valve.

HPLC Separation

The aflatoxins were isocratically separated on a RP-HPLC column with the described mobile phase containing water, methanol and acetonitrile. The flow rate was 1 mL per minute. The aflatoxins appeared to be base line resolved and eluted in the order $AfG_2 \rightarrow AfG_1 \rightarrow AfB_2 \rightarrow AfB_1$.

Post Column Derivatization

The KOBRA-cell ® settings were according to the specification of the manufacturer (see parameters below), while Figures 3.1 and 3.2 show the schematic HPLC setup for both systems.

KOBRA-cell ® settings:

Mobile phase according to previous section, containing
120 mg KBr and 350 μL HNO_3 ($c(HNO_3)$=4 mol/L) each litre.
Electrical current: 100 μA.
Reaction tubing with 40 cm and 0.5 mm inner diameter (equivalent to a reaction time of $\geq$ 4 seconds).

The addition of PBPB as bromination agent was performed with an auxiliary pulse-less HPLC pump. As a pressure and flow buffer, a Silica gel (Mesch 60 - 200) filled housing of an old plastic-polymer column (25 cm, 4.6 mm inner diameter) was used.

PBPB settings:

Mobile phase according to previous section.
Reagent solvent: 50 mg/L PBPB in water.
Void volume T-piece mixing chamber.
Flow-rates: 1 mL/min. (mobile phase) - 0.3 mL/min. (reaction solvent).
Reaction tubing with 60 cm and 0.5 mm inner diameter (equivalent to a reaction time of $\geq$ 4 seconds).

In addition, flow chart diagrams of the described procedures are given in Figures 3.3 to Figures 3.5.

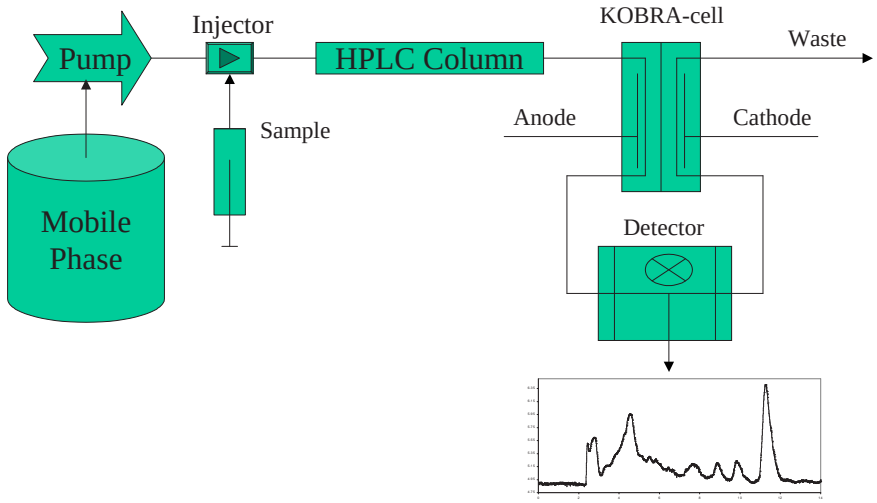


Figure 3.1: HPLC setup for KOBRA-cell ® derivatization

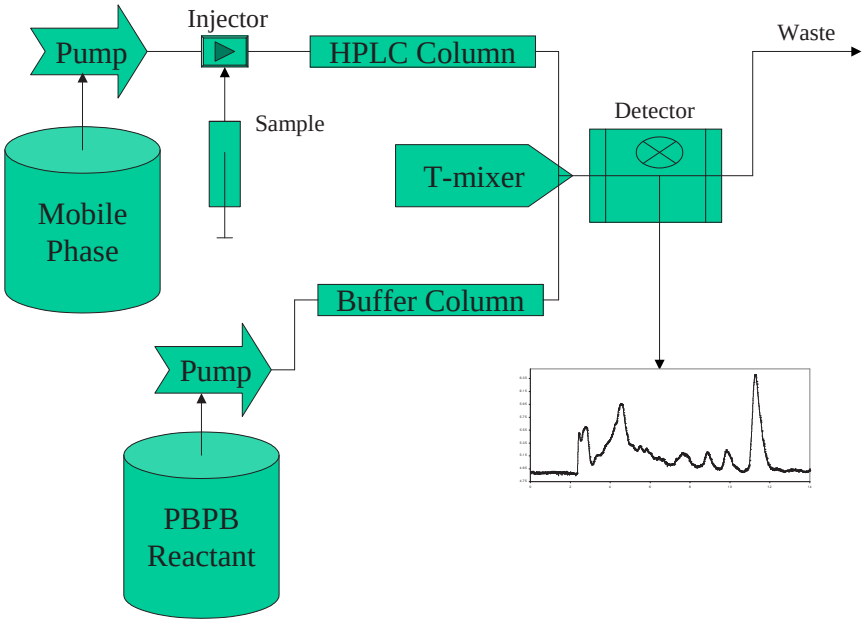


Figure 3.2: HPLC setup for PBPB derivatization

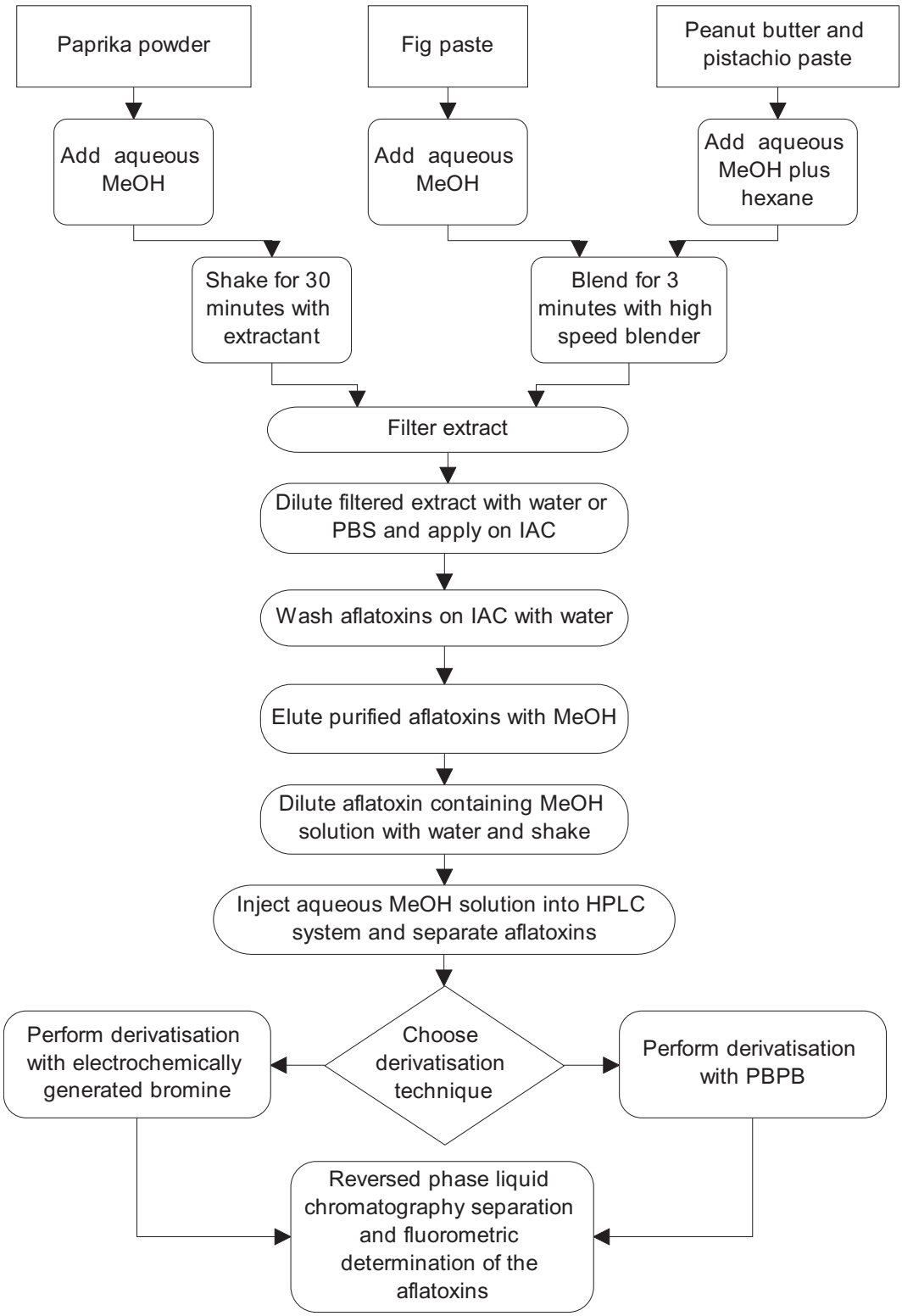


Figure 3.3: Flow chart - Determination of aflatoxin B_1 and total aflatoxins in paprika powder, fig paste, pistachio paste and peanut butter by HPLC

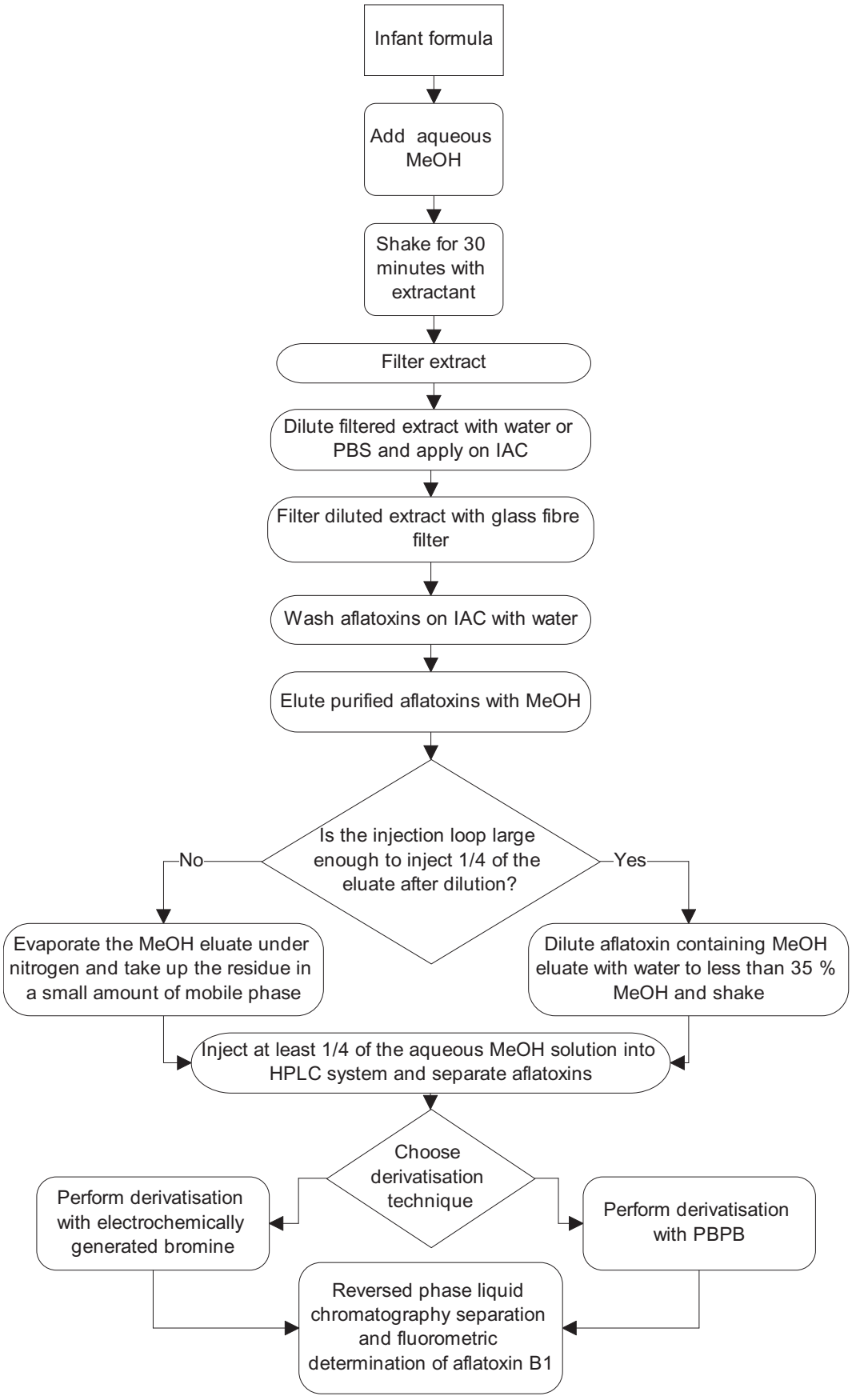


Figure 3.4: Flow chart - Determination of aflatoxin B_1 in infant formula by HPLC

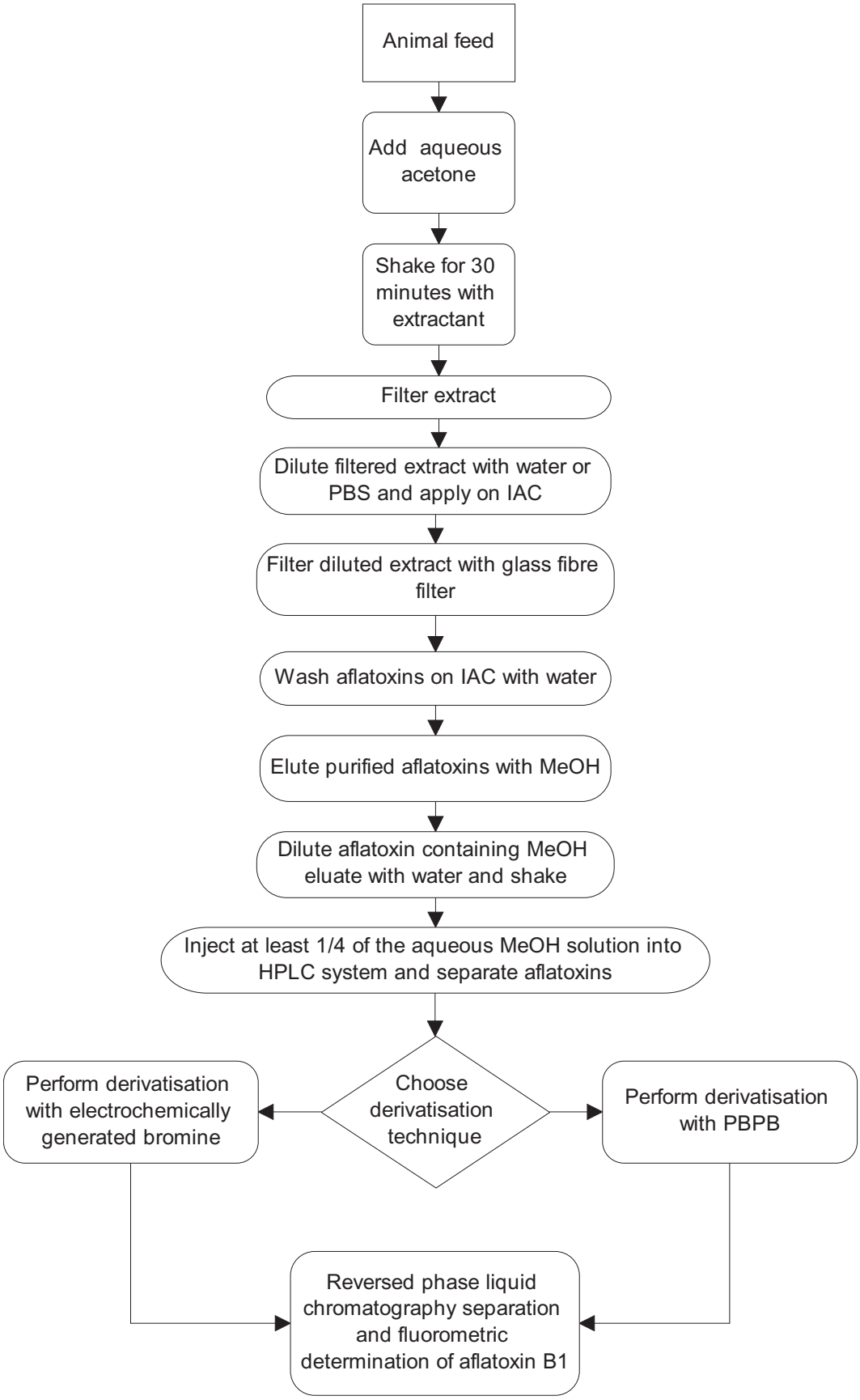


Figure 3.5: Flow chart - Determination of aflatoxin B_1 animal feedingstuff by HPLC

Fluorimetry

Aflatoxin B_1, B_2, G_1 and G_2 were separately dissolved in toluene:acetonitrile (9+1 [v/v]) to give solutions containing 10 g/ml of each aflatoxin. The exact concentration of aflatoxins in each solution was determined by recording the absorption curve between a wavelength of 330 nm and 370 nm in 1 cm quartz glass cells in a spectrometer with toluene/acetonitrile in the reference path. The mass concentration of each aflatoxin was determined according to:

$$C = \frac{A_{max} * M_i * 100}{E_l * d}$$

- C = Concentration of the Aflatoxin in [g/mL]
- A_{max} = Absorbance determined at the maximum of the absorption curve
- M_i = Relative molecular mass of each aflatoxin [in g/mol]
- E_l = Molar absorptivity of each aflatoxin in toluene/acetonitrile [in m^2/mol]
- d = Optical path length of the cell [in cm]

The molar masses and absorptivities for each aflatoxin are given in Table 3.1 and were derived from a recent study for the determination of aflatoxins in various solutions [166].

Aflatoxin	M_i **[g/mol]**	E_l [m^2/mol]
B_1	312	1930
B_2	314	2100
G_1	328	1640
G_2	330	1830

Table 3.1: Molar absorptivities of aflatoxins in toluene:acetonitrile (9+1 [v/v])

3.2.5 TLC Procedures

Application (*spotting*) of the the Aflatoxins on the TLC-plate

Exactly 100 μL of the re-dissolved aflatoxin solution (Chapter 3.2.3) were spotted on a silica-gel 60 TLC plate with a gas-tight micro litre syringe and dried with warm air.

TLC Separation

After application, a pre-run procedure was performed prior to the main separation. This was done with neat MeOH, which was allowed to run just over the applied analyte spots, concentrating all of the analyte into small bands. The plate was dried with warm air to assure that no MeOH residues remained on the TLC-plate. The aflatoxins were separated with the mobile phase in an unlined and unsaturated TLC tank in the dark.

Fluorescence Amplification

For fluorescence amplification the developed TLC-plates were sprayed with a solution of 10 % paraffin in *n*-hexane. The *n*-hexane was evaporated and the fluorescence was determined thereafter. A flowchart of the method procedures is given in Figure 3.6.

Quantification

Scans of the developed TLC-plates were performed with the CAMAG TLC-Scanner as reference in addition to the developed prototype systems and visual detection. A direct comparison of the semiconductor densitometer and the modified flat-board scanner was not performed, due to technical problems (mounting and dismounting procedures during development of both prototypes).

Visual Determination Visual detection of the aflatoxins was carried out by radiation of the aflatoxins with a UV-light source of 366 nm in the darkness. Spots from the samples and standards were compared for intensity and reported as »*smaller than*«, »*between*« or »*equivalent to*« the corresponding standards. Spots were marked[2] for documentation with a soft pencil after reporting of intensity.

[2]after finishing all densitometric measurements

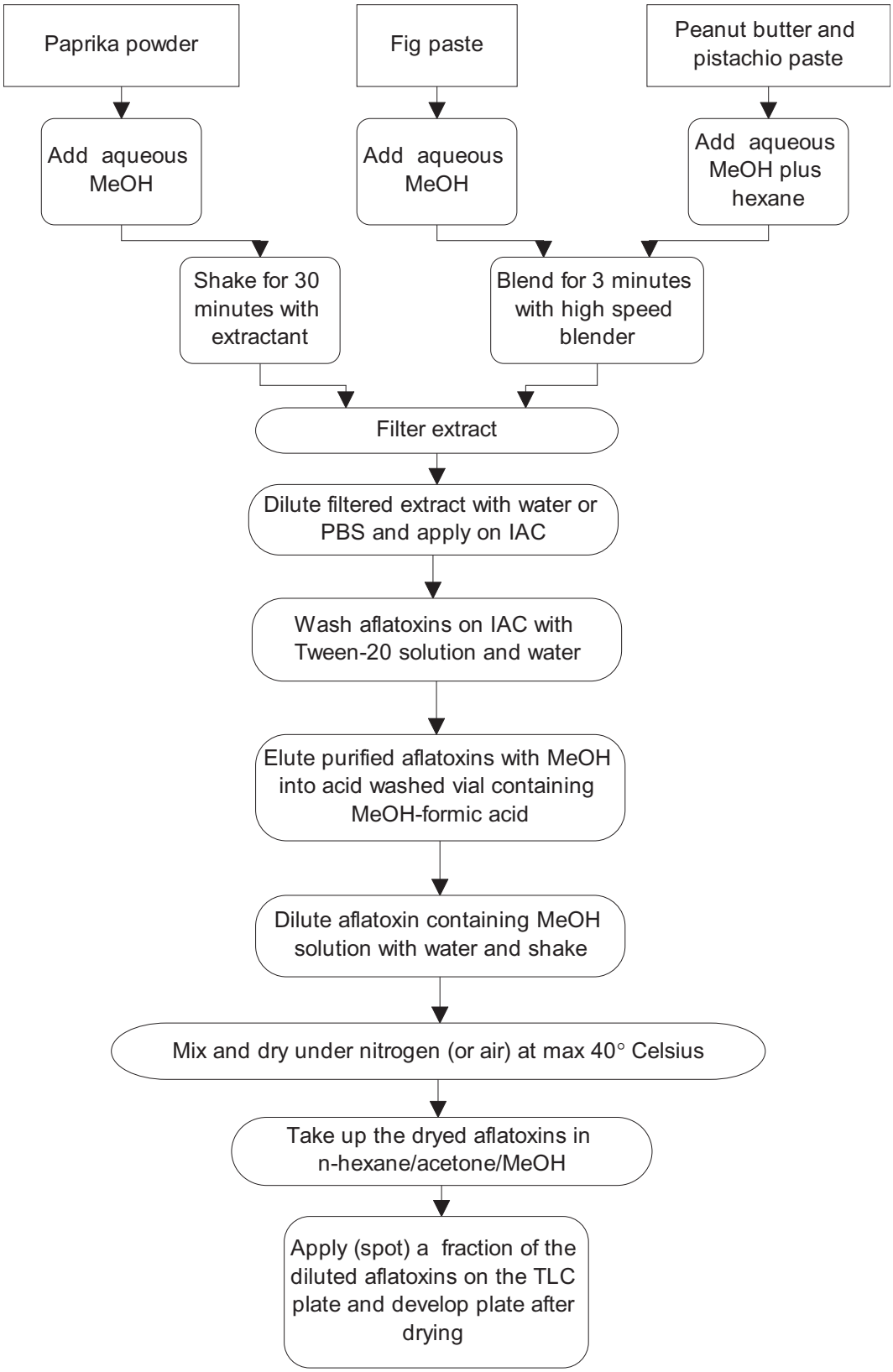


Figure 3.6: Flow chart - Determination of aflatoxins by TLC in paprika powder, fig paste, pistachio paste and peanut butter

CAMAG TLC-Scanner TLC-plates were scanned for fluorescence with an excitation wavelength of 366 nm (mercury lamp). The emitted fluorescence was filtered from the excitation with a cut-off filter of $\geq$ 418 nm. The densitometric scans were performed with the following parameters:

- scanwidth: 8 mm
- slitwidth: 0.2 mm
- data step resolution: 100 μm

Semiconductor Densitometer (SeBaDeC) The location of the aflatoxin spots was marked on the vertical sides of the TLC-plates[3] and the detector unit was moved horizontally along the corresponding aflatoxins. The detector unit was moved manually with a threaded bold support. Scans were performed with a data sampling rate of 23 points per second[4]. Previous to each scan the blank signal was adjusted to approximately 300 mV over an aflatoxin free region of the TLC-plate. The data was recorded and saved as a text-file[5]. The data was imported to a spread-sheet programme[6] and plotted into a diagram.

Measurements with the Modified Flat-board Scanner The modified flat-board scanner that was built in co-operation with the *Fraunhofer Institut für Optik und Feinmechanik* in Jena, Germany, was directly attached to a PC via the parallel port. The driver that came with the scanner (VistaScan 3.5.1) was installed on the PC under Windows98 and the image was scanned after a warm up phase of approximately 10 minutes in the advanced mode.

Thereafter the developed TLC plate was pre-viewed under a UV-lamp and the area that contained the aflatoxin spots was marked with a fluorescent felt pen. The plate was then positioned at an angle of 90^o on the image support (glass) of the scanner. Thus all spots with the same retention time were scanned at the same position along the UV-tube axis.

The TLC-plate was pre-viewed and the area containing the aflatoxins was selected according to the previously marked dimensions (inside the fluorescent felt pen corners).

[3]This was known from the scan with the CAMAG densitometer
[4]The data sample rate was selected with the program *MultiMet*
[5]tab-delimited numbers of the signal in mV
[6]Microsoft Excel97

TLC-plates were scanned with the device at a resolution of 300 dpi[7] and at 36-bit colour depth[8] and saved as an image file[9]. All automatic functions of the scanner driver were disabled during this scan and manual adjustments were made to increase contrast and saturation if necessary.

The evaluation of the image was performed with an image measurement software[10]. Therefore the colour channels of the image were extracted into single channels and only the blue-channel data was taken for further analysis[11]. To measure the aflatoxin spots the line intensity of a 71 pixels wide line was performed (the whole peak was covered). This line was positioned along the aflatoxin spots, covering all spots of the same aflatoxin type. The intensities were automatically transferred into a table and further processed with a spread-sheet program[12].

3.2.6 Salting Out Procedures

A volume of 100 ml of extractant was filled into a 100 ml volumetric cylinder. Sodium chloride or sucrose was added in portions of 0.5 g. The extractant was then shaken, until all solids were diluted or a layer separation occurred.

3.2.7 Dry Mass Determination (*Extract Residue*)

A portion of the filtrate (approximately 1 ml) was weighed into a small beaker. The dry mass was calculated after evaporation of the extractant at 102±2°C until a constant weight was obtained.

3.2.8 Water Determination

The water determination of extractants was performed by the Karl-Fischer method. An exactly measured amount of approximately 100 mg of the extractant was injected in the reacting chamber. Results were directly calculated by the Karl-Fischer device after calibration.

---

[7] dots per inch

[8] 12-bit per channel of Red, Green and Blue (resolution of 2048)

[9] TIFF-Format

[10] *SigmaScan*, *SigmaScan Pro* or *NHI image*

[11] the image had to be reduced to 8-bit grayscale since the software did not support higher values

[12] Microsoft Excel97

3.2.9 Homogeneity Testing

Homogeneity testing was performed at two stages during material preparation. At the 1st stage the material was tested for homogeneity after the required amount of test material was prepared (*bulk homogeneity*). After achieving a positive result for *bulk homogeneity* the material was packed in test material containers and the homogeneity was tested again between randomly chosen containers (*container homogeneity*).
For both homogeneity test series a fraction of 10 times 50 g (50 g = total content of a material container) was taken for analysis. These fractions were mixed again (or in the case of fig paste divided into small pieces of approximately 0.5 g) and split in two parts. All separated sub-fractions were analysed separately.

3.2.10 Statistical Tests

The statistical tests that were used during the collaborative trial studies are briefly described below. For detailed descriptions and exact deviations of these parameters the following documents may be used [107, 142, 143, 167]. It must be noticed that for the adoption of analytical methods several different parameters might be applied or defined. This holds true especially for the confidence levels that exist for statistical tests.

For example, the adoption of a method according to AOAC International[13] requires the use of the application of a probability level of P = 2.5% for Cochran and Grubbs outlier tests. In addition the maximum outliers in a study must not exceed 22.2%. Other organizations might indicate other probability levels or do not explicitly regulate the maximum amount of accepted outliers [167].

Outlier Testing

The outlier identification of collaborative trial data was performed according to the *AOAC harmonized protocol* for the design, conduct and interpretation of method-performance studies [52]. This procedure requires the application of the Cochran outlier test (1-tail, P = 2.5%) followed by the Grubbs outlier test (2-tail, P = 2.5%) for single and paired outliers. The *AOAC harmonised protocol* limits the outliers to maximum 2/9 of all results. Thus a collaborative trial must not exceed 22.2% identified outliers for validation. The outlier identification scheme was performed according to the AOAC harmonized protocol [52] (Figure 3.7).

[13] All statistical tests described here were according to AOAC statutes.

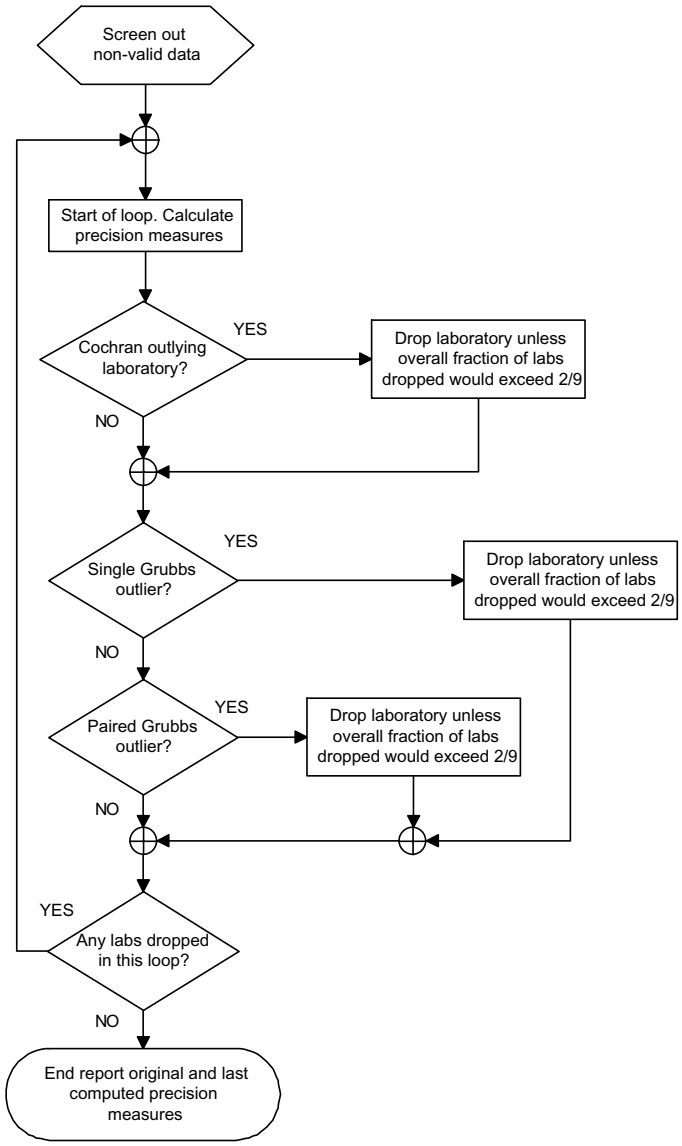


Figure 3.7: Flowchart for outlier removal according to AOAC harmonized protocol

Outlier test according to Cochran The outlier identification according to Cochran requires that the number of results from each laboratory is the same ($n_i = n \veebar i$).

In the first step the within-laboratory variance (s_i^2) of each laboratory set was determined. Then the sum of all the variance ($\sum_{i=1}^{m} s_i^2$) was determined, and the largest of the within-laboratory variances (s_{max}^2) was multiplied by 100, divided by ($\sum_{i=1}^{m} s_i^2$).

$$C = 100 \times \frac{s_{max}^2}{\sum_{i=1}^{m} s_i^2}$$

The C value was then compared with a given value in a table [52], or could be calculated (for $n_i = 2$)[14] with the function below. Values that exceeded the given values indicated outliers.

$$C_{max} = 77.1106e^{-0.1201m} + 50.2915e^{-0.017m}$$

Definition range: $\mathrm{m} \in \mathrm{N} \cap 4 \leq \mathrm{m} \leq 50$

Single outlier test according to Grubbs For the single outlier identification according to Grubbs, the mean value $\overline{x}$ of the results from each laboratory for identical samples was determined, followed by the calculation of the standard deviation s_i of these (between laboratory) *mean* values and the identification of the absolute maximum deviation value (Δx_i^*). Then the standard deviation of the set with the highest s_{H*} and with the lowest average s_{L*} removed was determined and the percentage decrease for the highest or the lowest removed average was determined:

$$100 \times [1 - \frac{s_{H*}}{s_i}] \qquad \text{and} \qquad 100 \times [1 - \frac{\mathrm{s_{L*}}}{\mathrm{s_i}}]$$

The larger of these percentage decreases in s_i (after removal of the highest or lowest value) indicated a single Grubbs outlier, if this decrease exceeds the corresponding value from a table [52]. Alternatively, the value for the single *One Highest Or One Lowest* outlier can be calculated for (m) number of laboratories:

$$G_{1H \bigcup 1L} = 150e^{-0.758486m} + 100.33e^{-0.176948m} + 31.5692e^{-0.0213795m}$$

Definition range: $\mathrm{m} \in \mathrm{N} \cap 4 \leq \mathrm{m} \leq 50$

[14] Blind duplicate values for each matrix/concentration level

Paired outlier test according to Grubbs Based on the Grubbs test for a single outlier, this test allows the identification of paired outliers for two cases; the *Two Highest Or Two Lowest* ($G_{2H \bigcup 2L}$) or *One Highest And One Lowest* ($G_{1H \bigcap 1L}$) outlier. The calculation for the *paired outlier* tests were performed similar to those of the single Grubbs outlier test (removal of the two highest or two lowest values respectively the highest and lowest value). However the values for the critical percentage drop must be taken from a different table [52] or can be calculated according to:

$$G_{2H \bigcup 2L} = 114.304e^{-0.186144m} + 43.6767e^{-0.0327465m} + 7.49216e^{(-1.7585E-11)m}$$

$$G_{1H \bigcap 1L} = 82.3751e^{-0.156859m} + 28.2065e^{-0.156873m} + 44.8292e^{-0.0193887m}$$

Definition range: m $\in$ N $\cap$ 4 $\leq$ m $\leq$ 50

Analysis of Variances (ANOVA) and F-test

The analysis of variance was used during homogeneity testing and was performed in order to determine that the *between sample variance* of the different fractions was not significantly different than the *within variance* of the single units (50 g test material). In addition the obtained variance had to be not significantly different than the analytical error that was previously established for the method (See chapter 2.2.1).

Every 10th sample container was taken from the sequence of finally packed material and was subjected to analysis. The number of the first glass from which the sampling started was randomly determined for each material respectively. As an example peanut butter with a content of 1 μg/kg AfB_1 was checked by taking the fourth, the 14^{th}, the 24^{th} - and so on - sample. In the first part of the homogeneity test the data were analysed by using the statistical technique ANOVA that allows the partitioning of the whole variance into the individual components of variability. In this study 10 samples were analysed in duplicate: from each sample 2 sub-samples were analysed. Using the ANOVA technique the following variances can be calculated:

- Standard deviation$_{\text{Within-unit}} = \sqrt{Within - Group - Variance} = \sqrt{\sigma^2_{within}}$

Assuming that the homogeneity within the containers can be considered as negligible this component reflects the analytical error.

- Between-Group-Variance = $\sigma^2_{between}$
- Overall variance = $\sigma^2_{total} = \sigma^2_{between} + \sigma^2_{within}$
- Standard deviation$_{\text{Betweensamples}} = \sqrt{\frac{\sigma^2_{between}+\sigma^2_{within}}{2}} = \sigma_s$

The latter component reflects the heterogeneity of the material and can only be calculated if the F-test of the ANOVA indicates a significant difference of $\sigma^2_{between}$ and σ^2_{within}. The material can be regarded as sufficiently homogeneous if the variances mentioned above do not differ significantly from the variance of the repeatability of the method. This repeatability was determined in the in-house performance study. The equality of the variances was checked by using the F-test at the 95% confidence interval level.
The ANOVA test was performed using an internal Macro provided by Microsoft Excel.

t-test

The t-test was applied in addition to the ANOVA during the homogeneity testing in order to check for any drift in the results during analysis. The test comprises the following steps:

- The samples of each material were arranged in the chronological order in which they had been analysed making up a row of 20 values.
- The *mean* ($\overline{x}_1$) of the 1^{st}, 2^{nd} and 3^{rd} value and the *mean* ($\overline{x}_2$) of the 18^{th}, 19^{th} and 20^{th} value were calculated respectively. In case of a trend in the results the difference of these two values would be significant.
  - In order to check for a significant difference of the means the t-value was calculated according to the following equation:

    $$t_{calc} = \frac{|\overline{x}_1 - \overline{x}_2|\sqrt{3}}{STDEV}$$

    where SD is the standard deviation of the method determined in the in-house performance study.

  - The calculated t-value (t_{calc})was compared with the critical t-value (t_{crit}). If t_{calc} is within the range of $\pm t_{crit}$, the difference of the means is not significant indicating that no trend of the results can be observed.

Repeatability (r)

The repeatability[15] of a method is a figure that describes the relation between two single analytical obtained under the below described conditions and indicates the maximum deviation of two results with a certain probability (generally 95%). The *repeatability conditions* imply that results were obtained by:

- application of the same analysis method,
- analysis of identical test material (matrix and contamination level) and
- identical conditions (same operator, same equipment, shortest time frame possible)

The repeatability (r) is calculated from a probability dependent factor and the $Standarddeviation_{within-laboratory}$ (s_r) according to:

$$r = 2.83 * s_r$$

The factor of 2.83 reflects a probability of 95%. Other factors might be applied for a probability of 90% (2.32) or 99% (3.65)

$$s_r = \sqrt{\frac{1}{N-m}\sum_{i=1}^{m}\sum_{k=1}^{n(i)}(x_{ik}-\overline{x}_i)^2}$$

with:

- m = number of laboratories
- n = number of results from laboratory i
- N = number of all obtained results = $\sum_{i=1}^{m} n_i$

For the calculation of the repeatability *Excel*-worksheets[16] were used. These worksheets contained all necessary *macros* to determine the repeatability.

[15] derived from the results of an inter-laboratory study

[16] Courtesy of Ken Mathieson CSL, Food Science Laboratory, York, UK

Reproducibility R

The reproducibility (R) is a figure that describes the relation between two single analytical that were obtained under different conditions:

- different operators and

- different facilities (laboratory) and/or (time of analysis)

while the analysis method and the test material used must be the same. The reproducibility is calculated similar to the repeatability according to:

$$R = 2.83 * s_R$$

$$s_R = \sqrt{\frac{m}{N} s_Z^2 + \frac{N-m}{N} s_I^2}$$

The variances s_Z^2 and s_I^2 reflect the between respectively the within laboratory variances. Both figures are calculated according to:

$$s_Z^2 = \frac{1}{m-1} \sum_{i=1}^{m} n(i)(\overline{x}_i - \overline{\overline{x}})^2$$

$$s_I^2 = \frac{1}{N-m} \sum_{i=1}^{m} \sum_{k=1}^{n(i)} (x_{ik} - \overline{x}_i)^2$$

For the calculation of the reproducibility Excel-worksheets[17] were used. This worksheets contained all necessary *macros* to determine the reproducibility.

3.2.11 Photometry

For the colour determination of fig paste extracts[18] the spectra of a coloured fig paste extract was determined in the range of 200 - 800 nm in a 1 cm cuvette against the extractant as reference. The main absorption maximum was found to be at 410 nm.

This maximum was used for further investigations of the extract colour. In general the extracts were diluted with the extractant[19] to obtain absorption values of maximum 0.700 and were subsequently analysed.

[17] Courtesy of Ken Mathieson CSL, Food Science Laboratory, York, UK

[18] indicator for the extent of the heat treatment

[19] methanol-water (8+2)

Chapter 4

Summary

Aflatoxins are toxic metabolites that can occur in food and animal feed. Due to their hepatotoxic and carcinogenic properties they are regulated in a large number of countries. Recently the European Commission set new legal limits for aflatoxin B_1 and total aflatoxins [46]. To support the monitoring of these contaminants, the need for adequate (validated) analytical methods was expressed.

The main approach of this work was to develop new, adapt already existing, and finally validate HPLC and TLC methods. Main focus during this process was to put fast, efficient, robust, and user friendly procedures on the use of non toxic solvents. Due to the structure of this work, no general conclusions can be reached. Thus several single and independent conclusions will be drawn, while as a matter of fact, all given goals of this work were achieved successfully.

Concerning method development and validation (collaborative trial study), it can be concluded that robust, efficient and user friendly methods were achieved. The described methods were applicable for most relevant matrices such as fig paste, paprika powder, peanut butter, pistachio paste, animal feedingstuff, and infant formula, while only small deviations in the methods were required for adoption to the various matrices and target levels. These include the extraction solvent, the immunoaffinity clean-up and injection volume. The extraction of sample material was identified to be a critical procedure, since certain extractants, such as aqueous acetonitrile, can lead to false results for aflatoxin determination (recoveries of more than 150%). Subsequently, after drafting the methods, collaborative studies were conducted.

Collaborative trial study on paprika powder, pistachio paste, peanut butter, and fig paste For this HPLC method validation, excellent pre-

cision data was obtained for all matrices. Repeatability and reproducibility values were far below figures that were predicted through comparison with previous studies [145]. Thus spiked levels of 2.4 μg/kg and 9.6 μg/kg for total aflatoxins which included incorporation of amounts of 1.0 μg/kg and 4.0 μg/kg of aflatoxin B_1 respectively. Recoveries for total aflatoxins ranged from 71% to 92% with the corresponding recoveries for aflatoxin B_1 ranging from 82% to 109%. Based on results for spiked samples (blind pairs at two levels) as well as naturally contaminated samples (blind pairs at four levels including blank) the relative standard deviation for repeatability (RSD_r) ranged from 4.6% to 23.3% for total aflatoxins and from 3.1% to 20.0% for aflatoxin B_1. The relative standard deviation for reproducibility (RSD_R) ranged from 14.1% to 34.2% for total aflatoxins, and from 9.1% to 32.2% for aflatoxin B_1. The method showed acceptable within-laboratory and between-laboratory precision for all four matrices, as evidenced by HORRAT ratios, at the low levels of determination for both total aflatoxins and aflatoxin B_1.

Collaborative study on infant formula Concerning this collaborative study, samples were spiked at levels of 0.1 μg/kg and 0.2 μg/kg for aflatoxin B_1. Recoveries ranged from 101% - 92%. Based on results for spiked samples (blind pairs at two levels) as well as naturally contaminated samples (blind pairs at three levels) the relative standard deviation for the RSD_r ranged from 3.5% - 14%. The relative standard deviation for the RSD_R ranged from 9% - 23%.

Collaborative study on animal feedingstuff For this collaborative study samples were spiked at levels of 1.2 μg/kg and 3.6 μg/kg for aflatoxin B_1. Recoveries ranged from 74% - 157%. Based on results for spiked samples (blind pairs at two levels) as well as naturally contaminated samples (blind pairs at three levels) the relative standard deviation for the RSD_r ranged from 5.9% - 8.7%. The relative standard deviation for the RSD_R ranged from 17.5% - 19.6%.

All obtained precision data from these collaborative studies fulfilled the criteria for analytical methods that were given by the European Committee for Standardisation (CEN) [51]. Thus the work clearly showed that with care and attention to detail during the organization of the collaborative trial it was possible to achieve impressive performance characteristics even at low limits of detection.

TLC-method development Concerning the development of a novel TLC method that fully abstains from the use of any chlorinated or highly toxic

solvents, SIMILAR in-house performance data was obtained, as reported for the HPLC method. The sample preparation was derived from the previous procedures used for HPLC and contained an immunoaffinity clean-up step. Such a clean up procedure is still uncommon for TLC methods. Spotting solvents and mobile phases were developed on the base of non-chlorinated and non-toxic solvent mixtures. It was found that a spotting solvent based on *n*-hexane, acetone and methanol showed excellent properties, while a mobile phase based on a mixture of *tert*-butylmethylether, methanol and water sufficiently separated aflatoxin spots on normal silica gel TLC-plates.

Simplified TLC-densitometers Two new simple and low-cost alternatives for currently available TLC densitometers were described and evaluated: a miniaturized device based on a solid stated detector cell (SeBaDeC) and a modified office scanner. The first approach was found to be an inexpensive alternative to the currently used equipment. Due to the low power consumption of a novel UV-light source this SeBaDeC principle offers battery operation. Furthermore the lifetime of the light source is superior compared to mercury lamps or gas tubes.

The signal processing and data recording were based on inexpensive and commonly available electronic components. The performance data demonstrated that the SeBaDeC is suitable for the determination of aflatoxins at European regulatory limits of 2 μg/kg aflatoxin B_1 (respectively 4 μg/kg total aflatoxins) in combination with the previously described TLC method. However, at the current state of development, further improvements of the prototype in design of the cell and electronic circuits are still conceivable. However it is questionable if the effort of such improvements will meet the expectations, since further performance characteristics are not required for aflatoxin measurements. Other developments, such as the application of the SeBaDeC principle for the measurement of aflatoxins on Florisil tips are more promising, since this would allow a rapid determination of total aflatoxins (for screening purposes) without any chromatographic separation.

The approach by modification of an office scanner was found to be another interesting and user friendly alternative. In this approach the light tube was substituted by a commonly available UV-tube, while this excitation light was cut-off at the detector module with an high-pass filter, thus allowing only the fluorescence to be measured. For measurement, the resulting images were processed with imaging software. The sensitivity of this devices has been shown to be sufficient to determine aflatoxin amounts of $\geq$2 ng Scanner absolute.

With some limitations, both devices (Scanner and SeBaDeC) have shown

to compare with a commercially available scanner for the densitometric measurement of aflatoxins on TLC-plates.

Bibliography

[1] Sydenham EW, Shephard GS (1996) Chromatographic and allied methods of analysis for selected mycotoxins, in: Gilbert J (Editor), Progress in Food Contaminant Analysis, ISBN: 0-7514-0337-7

[2] Steyn PS (1998) The biosynthesis of mycotoxins. vue Méd Vét 149: 469-478

[3] Cole RJ, Cox RH (1981) Handbook of toxic fungal metabolites. Academic Press, New York ISBN: 0-12-179760-0

[4] Bullerman LB (1999) Mycotoxins, in: Robinson RK, Batt CA, Patel PD (Editors), Encyclopedia of food microbiology, Academic Press, ISBN: 0-12-227070-3

[5] Moss MO (1989) Mycotoxins of Aspergillus and other filamentous fungi. J Appl Bacteriol Symp Suppl 1989: 69S-81S

[6] Goldblatt LA (1969) Aflatoxin - Scientific Background, Control, and Implications, pp. 13-46 (Aflatoxin Formation by Aspergillus Flavus). Academic Press, New York

[7] Chitrangada D, Mishra HN (2000) Effects of aflatoxin B_1 detoxification on the physicochemical properties and quality of ground nut meal. Food Chem 70: 483-487

[8] Bhatnagar D, Lillehoj EB, Benett JW (1991) Biological detoxification of Mycotoxins, in: *Mycotoxins and Animal Foods* by Smith JE (Ed.). CRC-Press ISBN: 0-8493-4904-4

[9] European Commission (1994) Mycotoxins in Human Nutrition and Health (Document EUR 16048 EN). European Commission Bruxelles

[10] Rustom IYS (1997) Aflatoxin in food and feed: occurrence, legislation and inactivation by physical methods. Food Chem 59: 57-67

[11] International Agency for Research on Cancer (1993) IARC Monographs on the evaluation of carcinogenic risk to humans. Volume 56, IARC Working goup, World Health Organization, Lyon, France

[12] Marquardt H, Schäfer SG (1994) Lehrbuch der Toxikologie. BI-Wissenschaftsverlag, Mannheim, Germany

[13] Terao K, Ohtssubo K (1991) Biological Activities of mycotoxins: field and experimental mycotoxicosis, pp. 455-488 in: *Mycotoxins and Animal Foods* by Smith JE (Ed.), CRC Press ISBN: 0-8493-4904-4

[14] Hsu IC, Metcalf RA, Sun T, Welsh JA, Wang NJ, Harris CC (1991) Mutational hotspot in the p53 gene in human hepatocellular carcinomas. Nature 350: 427-428

[15] Bressac B, Kew M, Wands J, Ozturk M (1991) Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. Nature 350: 429-431

[16] van Egmond HP, Svensson UK, Fremy JM (1997) Mycotoxins, in: *Monograph on residues and contaminants in milk and milkproducts*. International Dairy Federation ISBN: 9-290-98025-8

[17] Muecke W (1999) Schimmelpilze, Vorkommen - Gesundheitsgefahren - Schutzmassnahmen. Ecomed ISBN: 3-609-68000-9

[18] Bhatnager D, Cleveland TE, Payne GA (1999) Aspergillus flavus, in: Robinson RK, Batt CA, Patel PD (Editors), Encyclopedia of food microbiology, Academic Press, ISBN: 0-12-227070-3

[19] Scott PM (1991) Methods of analysis for mycotoxins - an overview, in: Analysis of oilseeds, fats and fatty foods, by Rossel JB & Pritchard JLR (Ed.) ISBN: 1-85166-614-1

[20] Moss MO (1991) Economic importance of mycotoxins - recent incidences. Int Biodeterior 27: 195-204

[21] Bhat RV (1988) Mould deterioration of agricultural commodities during transit: problems faced by developing countries. Int J Food Microbiol 7: 219-225

[22] World Health Organization (1997) World health statistics quarterly - Food safety and foodborne disease. 50: 143-147

[23] Battalico A (1989) Presence, persistence and toxicity of mycotoxins in foods, pp. 51-60 in: *Proceedings of the International Symposium on: Food Safety: new methods for research and control*, by De Paoli E, Galli CL, Restani P (Ed.) Milan Fair, November 22-23, 1998 ISBN: 8821419231

[24] Pittet A (1998) Natural occurrence of mycotoxins in foods and feeds - an updated review. vue Méd Vét 149: 479-492

[25] Strange RN (1991) Natural occurrence of mycotoxins in groundnuts, cottonseed, soya, and cassava, in: *Mycotoxins and Animal Foods* by Smith JE (Ed.). CRC-Press ISBN: 0-8493-4904-4

[26] Bhat RV, Vasanthi S, Rao BS, Rao RN, Rao VS, Nagaraja KV, Bai RG, Prasad CAK, Vanchinathan S, Roy R, Saha S, Mukherjee A, Ghosh PK, Toteja GS, Saxena BN (1997) Aflatoxin B_1 contamination in maize samples collected from different geographical regions of India - a multicentre study. Food Addit Contam 14: 151-156

[27] Medina-Martinez MS, Martinez AJ (2000) Mould occurrence and aflatoxin B_1 and fumonisin B_1 determination in corn samples in Venezuela. J Agric Food Chem 48: 2833-2836

[28] Resnik S, Neira S, Pacin A, Martinez E, Apro N, Latreite S (1996) A survey of the natural occurrence of aflatoxins and zearalenone in Argentine field maize: 1983-1994. Food Addit Contam 13: 115-120

[29] Bhat RV, Vasanthi S, Rao BS, Rao RN, Rao VS, Nagaraja KV, Bai RG, Prasad CAK, Vanchinathan S, Roy R, Saha S, Mukherjee A, Ghosh PK, Toteja GS, Saxena BN (1996) Aflatoxin B_1 contamination in groundnut samples collected from different geographical regions of India - a multicentre study. Food Addit Contam 13: 325-331

[30] Dahvan AS, Choudary MR (1995) Incidence of aflatoxins in animal feedstuffs: a decade's scenario in India. JAOAC 78: 693-698

[31] Scudamore KA, Hetmanski MT, Chan HK, Collins S (1997) Occurrence of mycotoxins in raw ingredience used for animal feeding stuffs in the United Kingdom in 1992. Food Addit Contam 14: 157-173

[32] Arim RH, Ferolin CA, Dumada-Ug LM (1995) Comparison of three methods for the determination of aflatoxins in copra meal. Food Addit Contam 12: 415-420

[33] Pohland AE (1993) Mycotoxins in rewiev. Food Addit Contam 10: 17-28

[34] Abdulkadar AHW, Al-Ali A, Al-Jedah J (2000) Aflatoxin contamination in edible nuts imported in Qatar. Food Control 11: 157-160

[35] Scholten JM, Spanier MC (1996) Determination of aflatoxin B_1 in pistachio kernels and shells. JAOAC 79: 1360-1364

[36] Pinto VEF, Vaamonde G, Brizzio SB, Apro N (1991) Aflatoxin production in soybean varieties grown in Argentina. J Food Prot 54: 542-545

[37] Mühlemann M, Lüthy J, Hübner P (1997) Mycotoxin contamination of food in Ecuador. Mitt Gebiete Lebensm Hyg 88: 474-496

[38] Piñeiro MS, Dawson R, Costarrica ML (1996) Monitoring programme for mycotoxin contamination in Uruguayan food and feeds. Natural Toxins 4: 242-245

[39] Hussain M, Vojir F (1993) Stichprobenplan für die Abnahmeprüfung beim Import getrockneter Feigen. Dtsch Lebensm Rundsch 89: 379-383

[40] Takahashi T (1993) Aflatoxin contamination in nutmeg: analysis of interfering TLC spots. J Food Sci 58: 197-198

[41] Shamsuddin ZA, Khan MA, Khan BA, Ahmad MA, Ahmad A (1995) Contamination of red chilli with aflatoxin B_1 in Pakistan. Mycotoxin Res 11: 21-24

[42] Patel S, Hazel CM, Winterton AGM, Mortby E (1996) Survey of ethnic foods for mycotoxins. Food Addit Contam 13: 833-841

[43] Maxwell SM, Apeagyei F, de Vries HR, Mwanmut DD and Hendrickse RG (1989) Aflatoxins in breast milk, neonatal cord blood and sera of pregnant woman. Tocicol Rev 8: 19-29

[44] Weidenbörner M (1999) Lebensmittelmykologie. Behr's Verlag, Hamburg Germany ISBN: 3-86022-457-3

[45] Food and Agriculture Organization of the United Nations (1995) Worldwide regulations for mycotoxins 1995 - A compendium, FAO Food and Nutrition Paper no. 64. FAO, Rome

[46] Commission Directive 98/1525/EC of 16th July 1999 amending Directive 97/194/EC on aflatoxins in food and Commission Directive 1999/39/EC of 6 May 1999 amending Directive 96/5/EC on processed cereal-based foods and infant formulae for infants and young children, Official Journal of the European Communities L124/8

[47] Commission Decision 97/613/EC of 8 September 1997 on the temporary suspension of imports of pistachios and certain products derived from pistachios originating in or consigned from Iran. Official Journal L 248, 11.09.1997

[48] Commission Decision 1999/356/EC of 28 May 1999 on the temporary suspension of imports of peanuts and certain products derived from peanuts originating in or consigned from Egypt. Official Journal L 139, 02.06.1999

[49] Wyatt RD (1991) Poultry, in: *Mycotoxins and Animal Foods* by Smith JE (Ed.). CRC-Press ISBN: 0-8493-4904-4

[50] Hansen RB, Norman KL (1999) Economic importance of aflatoxin to the Australian peanut industry, in: Dietzgen RD (Editor) Elimination of aflatoxin contamination in peanut - ACIAR Proceedings No. 89. Australian Centre for International Agricultural Research, Canberra. ISBN: 1-86320-270-6

[51] European Committee for Standardization (1996) Documents prEN-ISO 15141-1 and prEN-ISO 15141-2 on Food analysis, Horizontal methods - Criteria Analytical methods for mycotoxins, Brussels, Belgium

[52] William Horwitz, (1995) Protocol for the design, conduct and interpretation of method-performance studies (Technical report). Pure & Appl Chem 67: 331-343

[53] Coker RD, Jones BD (1988) Determination of mycotoxins, in: Food Science & Technology - A series of monographs, HPLC in food analysis. by R. Macrae (Ed.) ISBN: 0-12-464781-2

[54] Betina V (1989) Chromatographic methods as tools in the field of mycotoxins. J Chrom 477: 187-233

[55] Scott PM (1993) Recent developments in methods of analysis for mycotoxins in foodstuffs. Trends in Anal Chem 12: 373-381

[56] Ikins WG (1991) Modern methods of analyzing mycotoxins in foods, in: Instrumental methods for quality assurance in food, by DYC Fung & RF Matthews (Ed.) Marcel Dekker ISBN: 0-82478-278-X

[57] Leitao J, Bailly J-R, de Saint Blanquat G (1990) Determination of mycotoxins in grains and related products,

in: Food analysis by HPLC, by LML Nollet ISBN: 0-8247-8623-8

[58] van Rhijn JA, Viveen J, Tuinstra LGMTh (1992) Automated determination of aflatoxin B_1 in cattle feed by two-column solid phase extraction with on-line high-performance liquid chromatography. J Chrom 592: 265-269

[59] Official Methods of Analysis of AOAC International (1995) Natural toxins Vol II Chapter 47: 1-35

[60] Horwitz W, Albert R, Nesheim S (1993) Reliability of mycotoxin assays - an update. JAOAC Int 76: 461-491

[61] Scott PM, Trucksess MW (1997) Application of Immunoaffinity Columns to Mycotoxin Analysis (Review). JAOAC 80: 941-949

[62] Kok WTh, van Neer ThCH, Traag WA, Tuinstra LGMTh (1986) Determination of aflatoxins in cattle feed by liquid chromatography and post-column derivatization with electrochemically generated bromine. J Chromatogr 367: 231-236

[63] Roos AH, van der Kamp HJ, Marley EC (1997) Advantages of Immunoaffinity Columns compared to Florisil/C18 Columns for the Determination of Aflatoxins in Animal Feed. Mycotoxin Research 13: 2-10

[64] van Egmond HP, Heisterkamp SH, Paulsch WEE (1991) EC-collaborative study on the determination of aflatoxin B_1 in animal feeding stuffs. Food Addit Contamin 8: 17-29

[65] Patey AL, Sharman M, Gilbert J (1990) Determination of aflatoxin B_1 levels in peanut butter using an immunoaffinity column cleanup procedure: interlaboratory study. Food Addit Contam 7: 515-520

[66] Patey AL, Sharman M, Gilbert J (1991) Liquid-chromatographic determination of aflatoxin levels in peanut butters using an immunoaffinity column cleanup

method: international collaborative trial. JAOAC 74: 76-81

[67] Worner FM, Patey AL, Wood R (1992) Determination of the levels of aflatoxin in peanut butter using the aflaprep immunoaffinity column clean-up procedure: collaborative trial. J Assoc Publ Analysts 28: 1-10

[68] Trucksess MW, Stack ME, Nesheim S, Page SW, Albert RH, Hansen TJ, Donahue KF (1991) Immunoaffinity column coupled with solution fluorimetry or liquid chromatography post-column derivatization for determination of aflatoxins in corn [maize], peanuts and peanut butter: collaborative study. J Assoc Off Anal Chem 74: 81-88

[69] Standards Measurements and Testing Programme. Dedicated Call for Proposals (95/C 148/07). The Validation of analytical methods to determine the content of aflatoxin, ochratoxin, patulin in foodstuffs of vegetable origin. SMT4-CT96-2045 Project

[70] Beonke A (1998) Method validation for mycotoxin determination in food and feedstuffs. Trends in analytical chemistry 17: 10-16

[71] Crosby NT (1984) Review of current and future analytical methods for the determination of mycotoxins, Food Add Contam 1: 39-44

[72] Eppley RM (1966) A versatile procedure for assay and preparatory separation of aflatoxins from peanut butter products, J. Assoc. Off. Anal. 49, 1218-1223

[73] Goto T, Manabe M, Matsuura S (1979) Application of HPLC to the analysis of aflatoxins in foods and feeds - Preparation of sample and column. Agric Biol Chem 43: 2591-2592

[74] Robertson jr JA, Louise SL, Cucullu AF, Goldblatt LA (1965) Assay of aflatoxin in peanuts and peanut products using acetone-hexane-water for extraction. J Amer Oil Chem Soc 42: 467-471

[75] Thean JE, Lorenz DR, Wilson DM, Rodgers K, Gueldner RC (1980) Extraction, Cleanup, and Quantitative Determination of Aflatoxins in Corn. J Assoc Anal Chem 63: 631-633

[76] Yin L (1969) Note on acetonitrile as an extracting solvent for aflatoxins, J. Assoc. Off. Anal. 52, 880

[77] Bradburn N, Coker RD, Blunden G (1995) A comparative study of solvent extraction efficiency and the performance of immunoaffinity and solid phase columns on the determination of aflatoxin B_1. Food Chem 52: 179-185

[78] Egana JA, Candlish AAG (1993) Immunoaffinity column chromatography for aflatoxins: assessment with acetone/water extraction procedure, in: Scudamore J (Editor) Proceedings of a UK Workshop held on 21.-23. April 1993 at Brunel University on Occurrence and significance of myctoxins. pp 217-221

[79] Garner RC, Whattam MM, Taylor PJL, Stow MW (1993) Analysis of United Kingdom spices for aflatoxins using an immunoaffinity column clean-up procedure followed by high-performance liquid chromatographic analysis and post-column derivatization with pyridinium bromide perbromide. J Chromatogr 648: 485-490

[80] Patey AL, Sharman M, Gilbert J (1991) Liquid Chromatographic Determination of Aflatoxin Levels in Peanut Butter Using an Immunoaffinity Column Cleanup Method: International Collaborative Trial. JAOAC 74: 76-81

[81] RHONE-POLENC DIAGNOSTICS (1996) Aflaprep-Brochure. Rhone-Polenc Diagnostics, Glasgow, UK

[82] Roch OG, Blunden G, Coker RD, Nawaz S, (1992) Development and validation of a solid phase extraction/HPLC method for the determination of aflatoxins in groundnut meal. Chromatographia 33: 208-212

[83] Stroka J, Anklam E, Joerissen U, Gilbert J (2000) Immunoaffinity column cleanup with liquid chromatography using post-column bromination for determination

of aflatoxins in peanut butter, pistachio paste, fig paste, and paprika powder: collaborative study. JAOAC Int 83: 320 - 340

[84] Werner, G., 1991, Bestimmung der Aflatoxine in Futtermitteln nach selektiver Reinigung an einer Immunoaffinitaetssaeule, Agribiological Research, 44 (4), 289-298

[85] Sharman M, Gilbert J (1991) Automated aflatoxin analysis of food and animal feeds using immunoaffinity column clean-up and high-performance liquid chromatographic determination. J Chromatography 543: 220-225

[86] Chang HHL, de Fries JW, Hobbs WE (1979) Comparative study of two methods for extraction of aflatoxin from peanut meat and peanut butter. JAOAC 62: 1281-1284

[87] Shotwell OL, Goulden, ML (1977) Aflatoxin: comparison of analyses of corn by various methods. JAOAC 60: 83-88

[88] Peusch M, Mueller-Seitz E, Petz M, Mueller A, Anklam E (1997) Extraction of capsaicinoids from chillies (Capsicum frutenscens L.) and paprika (Capsicum annuum L.) using supercritical fluids and organic solvents. Z Lebensm Unters Forsch A 204: 351-355

[89] Jones BD (1972) Methods of aflatoxin analysis (G70). The Tropical Products Institute, London.

[90] Traag WA, van Trijp JMP, Tuinstra LGMTh (1987) Sample clean-up and post-column derivatization for the determination of aflatoxin B_1 in feedstuffs by liquid chromatography. J Chromatogr 396: 389-394

[91] Jain A, Hatch R (1980) Determination of Aflatoxins in Compound Feedstuffs Containing Citrus Pulp. J Assoc Off Anal Chem 63: 626-630

[92] Tuinstra LGMTh ,Verhuelsdonk CAH, Bronsgeest JM, Paulsch WE (1975) Aflatoxin B_1 in compound Feedstuffs

containing Citrus Pulp. Procedures for Screening and semi-quantitative Determination. Netherlands J Agric Sci 23: 10-17

[93] Betina V (1993) in: Chromatography of mycotoxins - techniques and applications. J Chromatogr library 54: 141-251

[94] Dyson N (1998) Chromatographic Integration Methods, Second Edition. The Royal Society of Chemists, UK ISBN: 0-85404-510-4

[95] Meyer VR (1992) Praxis der Hochleistungs - Flüssigkeitschromatographie. ISBN: 379355452X

[96] Commission Directive 87/410/EC of 14th July 1987, laying down the methods to be used for detection residues of substances having a hormonal action and of substances having a thyrostatic action, Official Journal of the European Communities L223/18

[97] Kussak A, Andersson B, Andersson K (1993) Automated sample clean-up with solid-phase extraction for the determination of aflatoxins in urine by liquid chromatography. J Chrom 616: 235-341

[98] Beaver RW (1990) Effects of injection solvent and mobile phase on efficiency in reversed-phase liquid chromatographic determination of aflatoxin M_1. JAOAC 73: 69-70

[99] Meyer V (1999) Fallstricke und Fehlerquellen der HPLC in Bildern. Wiley-VCH ISBN: 3-527-29794-4

[100] Takahashi DM (1977) Reversed-phase high performance liquid chromatographic analytical system for aflatoxins in wines with fluorescence detection. J Chrom 131: 147-156

[101] Joshua H (1993) Determination of aflatoxins by reversed-phase high performance liquid chromatograhpy with post-column in-line photochemical derivatization and fluorescence detection. J Chrom 654: 247-254

[102] Cepeda A, Franco CM, Fente CA, Vázquez BI, Rodriguez JL, Prognon P, Mahuzier (1996) Postcolumn excitation of aflatoxins using cyclodextrins in liquid chromatography for food analysis. J Chrom A 721: 69-74

[103] Sherma J (2000) Thin-layer Chromatography - The underappreciated analytical alternative. Inside Laboratory Management (AOAC Int) Jan. 2000

[104] Leming L, Zhang J, Wang P, Wang Y, Chen J (1998) Thin-layer chromatography of mycotoxins and comparison with other chromatographic methods. J Chromatogr A 815: 3-20

[105] Prosek M (2000) My favorite separation method, in: *Planar Chromatography 2000*, Proceedings of the International Society for Planar Separations in Lillafüred, Hungary.

[106] Kaiser RE (1986) Chromatographic Methods - Planar Chromatography vol. 1. Dr. Alfred Huethig Verlag, Heidelberg, Basel ISBN: 3-7785-0780-X

[107] Amtliche Sammlung von Untersuchungsverfahren nach paragraph 35 LMBG (1999) Nachweis und Bestimmung der Aflatoxine B_1, B_2, G_1 und G_2 in Lebensmitteln. Vol I/1 00.00-2

[108] Schweizerisches Lebensmittelbuch (1994) Toxische Stoffe natürlichen Ursprungs Vol VI.

[109] Montreal Protocol
http://www.unepie.org/ozat/protocol/main.html

[110] Carvajal M, Mulholland F, Carner CR (1990) Comparison of the Easi-Extract immunoaffinity concentration procedure with the AOAC CB method for the extraction and quantitation of aflatoxin B_1 in raw ground unskinned peanuts. J Chromatogr 511: 379-383

[111] Duncan K, Scott K, Zabe N, Kohn B, Prioli R (1998) Improved fluorometric and chromatographic methods for the quantification of fumonisins B_1, B_2 and B_3. J Chromatogr A 815: 41-47

[112] Kruger SC, Kohn B, RAmsey CS, Prioli R (1999) Rapid immunoaffinity-based method for determination of zearalenone in corn by fluorometry and liquid chromatography. JAOAC Intl. 82: 1364-1368

[113] Beaver R W (1990) Degradation of aflatoxins in common HPLC solvents. J High Res Chromatogr 13: 833-835

[114] Andrellos PJ, Beckwith AC, Eppley RM (1967) Photochemical changes of aflatoxin B_1. JAOAC 50: 346-350

[115] Coker DR, Jewers K, Tomlins KI, Blunden G (1988) Development of rapid methods for the analysis of aflatoxin in agricultural produce (I) Evaluation of instrumentation used for high-performance thin-layer chromatography of aflatoxins. Chromatographia 25: 875-880

[116] Kozloski RP (1981) Novel thin-layer chromatographic techniques for aflatoxin analysis. JAOAC 64: 1263-1264

[117] Hahn-Deinstrop E (1998) Dünnschichtchromatographie, Praktische Durchführung und Fehlervermeidung. Wiley-VCH ISBN: 3-527-28873-2

[118] Pons WA, Robertson jr. JA, Goldblatt LA (1966) Objective fluorometric measurement of aflatoxins on TLC plates. JAOCS 43: 665-669

[119] Trager WT, Stoloff L, Campbell AD (1964) A Comparison of Assay Procedures for Aflatoxin in Peanut Products. JAOAC 47: 993-1001

[120] Friedlander A, Gonen M (1970) Use of Polyamide Thin Layer Chromatography for Detection of Aflatoxins. Israel J Chem 8: 87-91

[121] Miskovic D (1980) Chromatographic analysis of mycotoxins on thin layers of rice starch. Chromatographia 13: 342-344

[122] Lee H, Ling KJ (1967) Application of Polyamide impregnated Film Thin Layer Chromatography for the Detection of Aflatoxins. Formosan Medical Assoc 66: 92-100

[123] Beljarrs PR, Fabry FHM (1972) Quantitative fluorodensitometric measurements of Aflatoxin B_1 with a Flying Spot Densitometer, I. Fluorodensitometric Study of the Behavior of Aflatoxin B_1 Standard Spots on different Types of Silica Gel. JAOAC 55: 775-780

[124] Heathcore JG, Hibbert JR (1975) Improvements in the thin-layer chromatography of natural products I. Thin-layer chromatography of the aflatoxins. J Chromatogr 108: 131-140

[125] Liang Y, Baker ME, Gilmore DA, Denton MB (1996) Evaluation of commercial silica-gel HPTLC-plates for quantitative fluorescence analysis. J Planar Chromatography 9: 247-253

[126] Engstrom WG (1969) New Solvent Systems for Thin-Layer Chromatography of Aflatoxins. J Chromatogr 44: 128-132

[127] Shi CN, Marth EH (1969) Improved Procedures for Measurement of Aflatoxins With Thin Layer Chromatography and Fluorometry. J Milk & Food Technol 32: 213-217

[128] Singh C (1970) Thin - Layer Chromatography of Aflatoxins. Anal Biochem 7: 568-569

[129] Stubblefield RD, Shannon GM, Shotwell OL (1969) Aflatoxins: Improved Resolution by Thin Layer Chromatography. JAOAC 52: 669-672

[130] Tomlins KI, Jewers K, Coker DR, Nagler JM (1989) A bi-directional HPTLC Development Method for the Detection of Low Levels of Aflatoxin in Maize Extracts. Chromatographia 27: 49-52

[131] Waltking A, Bleffert G, Fogerty R (1973) A new Benzene-Ethanol-Water Solvent System for TLC Separation of Aflatoxins. J Am Oil Chem Soc 50: 424-425

[132] Issaq HJ, Cutchin W (1981) A guide to thin-layer chromatographic systems for the separation of aflatoxin B_1, B_2, G_1 and G_2. J Liquid Chrom 4: 1087-1096

[133] Velasco J (1969) A simplified procedure for the determination of aflatoxin B_1 in cottonseed meals. JAOCS 46: 105-107

[134] Nesheim S (1969) Conditions and techniques for thin layer chromatography of aflatoxins. J AOCS 46: 335-338

[135] Shepherd MJ, Gilbert J (1981) . Food Addit Contam 1: 325-335

[136] Francis jun OJ, Kirschenheuter GP, Ware GM, Carman AS, Kuan SS (1988) β-Cyclodextrin post-column fluorescence enhancement of aflatoxins for reversed-phase liquid-chromatographic determination in corn [maize]. JAOAC 71: 725-728

[137] Jork H, Funk W, Fischer W, Wimmer H (1994) Thin-layer chromatography: Reagents and detection methods Vol. 1b. Verlag Chemie, Weinheim ISBN: 3-527-28205-X

[138] Zennie TM (1984) Instrumental HPTLC of aflatoxins: Fluorescence enhancement by corn free fatty acids. J Liquid Chrom 7: 1383-1391

[139] Software package: *Methodenvalidierung in der Analytik* (1999) Novia GmbH, Saarbruecken, Germany

[140] Beckwith AC, Stoloff L (1968) Fluorodensitometric measurement of aflatoxin thin-layer chromatograms. JAOAC 51: 602-608

[141] Commission Directive 98/53/EC of July 1998 laying down the sampling methods and methods of analysis for the official control of the levels for certain contaminants in foodstuffs. Official Journal fo the European Communities L 201/93 from 17.07.1998

[142] Youden WJ, Steiner EH (1975) Statistical Manual of the Association of Official Analytical Chemists. AOAC, Gaithersburg ISBN: 0-935584-15-3

[143] AOAC Int (1997) Associate referee's manual on development, study, review, and approval process. AOAC internal publication.

[144] Pemberton AD, Simpson TJ (1991) The Chemical Degradation of Mycotoxins, in: *Mycotoxins and Animal Foods* by Smith JE (Ed.). CRC-Press ISBN: 0-8493-4904-4

[145] Horwitz W, Albert R (1991) Performance characteristics of methods of analysis used for regulatory purposes. JAOAC 74: 718-744

[146] Cunniff P (Editor)(1995) Official Methods of Analysis, 16th Edition, Vol II. AOAC International, Arlington

[147] Peterson R, Ciegler A, Hall H (1967) Densitometric measurement of aflatoxin. J Chrom 27: 304-307

[148] Dikens J, Mcclure W F, Whitaker T B (1980) Densitometric equipment for rapid quantitation of aflatoxins of thin-layer chromatograms. J American Oil Chem Soc 57: 205-208

[149] Pons WA (1971) Evaluation of reflectance fluorodesitometry for measuring aflatoxins on thin-layer plates. J Assoc Off Anal Chem 54: 870-873

[150] Pons W, Robertson J A, Goldblatt L (1968) Objective fluorometric measurement of aflatoxins on TLC-plates. J American Oil Chemists Society 43: 665-669

[151] Robertson JA, Pons WA (1968) Solid State Fluorescence emission of aflatoxins on silica gel. J. AOAC 51: 1190-1192

[152] Toth L, Tauchmann F, Leistner, L (1970) Quantitativer Nachweis der Aflatoxine durch photofluorometrische Bestimmung direkt von Duennschichtplatten. Fleischwirtschaft 50: 1235-1237

[153] Whitaker TB, Dickens JW, Slate AB (1980) Computerized system to quantify aflatoxin using thin-layer chromatography. Peanut Science 17: 96-100

[154] Ayres JL, Sinnhuber RO (1966) Fluorodensitometry of aflatoxins on thin-layer plates. J American Oil Chem Soc 43: 423-424

[155] Berman MR, Zare RN (1975) Laser fluorescence analysis of chromatograms: Sub-nanogram detection of aflatoxins. Anal Chem 47: 1200-1201

[156] Bicking MKL, Knisley RN, Svec HJ (1983) Determination of aflatoxins in air samples of refuse-derived fuel by thin layer chromatography with laser induced fluorescence spectrometric detection. J Anal Chem 55: 200-204

[157] Ommenetto N (1998) Role of lasers in analytical atomic spectroscopy: where, when and why - Plenary Lecture. J Anal Atomic Spec 13: 385-399

[158] Prosek M (1997) Validation of quantitative TLC using digital video camera, in: Proceedings of the 9^{th} international symposium on instrumental planar chromatography. Interlaken, Swizerland 9.-11. April 1997

[159] Vovk I (2000) CCD sensors in planar chromatograph, in: *Planar Chromatography 2000*, Proceedings of the International Society for Planar Separations in Lillafüred, Hungary.

[160] Ryer A (1997) Light Measurement Handbook (International Light - iL) http://www.intl-light.com/handbook/

[161] Schommers A (1994) Elektronik gar nicht schwer - Experimente mit Optoelektronik. Band 4: 67-77 Elektor-Verlag GmbH Aachen, Germany ISBN: 3-928051-03-2

[162] Roithner Lasertechnik (2000) Data sheet for the photodiode EP-440. Roithner Lasertechnik, Fleischmanngasse 9, 1040 Vienna, Austria.

[163] Beuth K (1997) Bauelemente (Elektronik 2). Vogel Buchverlag, Wuerzburg, Germany ISBN: 3-8023-1713-0

[164] Schmidt WD (1997) Sensorschaltungstechnik (Elektronik 8). Vogel Buchverlag, Wuerzburg, Germany ISBN: 3-8023-1574-X

[165] Mocq F (1997) Multimet. Elektor 313: 52-53

[166] Nesheim S (1999) Molar absorptivities of aflatoxins B_1, B_2, G_1 and G_2 in acetonitrile, methanol, and toluene-acetonitrile (9+1) (Modification of AOAC Official Method 971.22): Collaborative Study. JAOAC Int 82: 251-258

[167] International Organization for Standartization (1998) ISO Standard 5725 - Accuracy (trueness and precison) of measurement methods and results. ISO, Genevre

[168] Dirksen AJ (1987) Elektronische Messinstrumente - Die Theorie des korrekten Messens. 235-245 Elektor-Verlag GmbH Aachen, Germany

[169] Kussak A, Andersson B, Andersson K (1995) Determination of aflatoxins in airborne dust from feed factories by automated immunoaffinity column clean-up and liquid chromatography. J Chrom 708: 55-60

[170] Dorner JW (1998) Chromatographic Analysis of Mycotoxins pp 113-168, in: *Chromatographic Analysis of Environmental and Food Toxicants* by Takayuki Shibamoto (Ed.) Marcel Dekker ISBN: 0-824-70145-3

[171] Samarajeewa U (1991) *In Situ* degradation of mycotoxins by physical methods, in: *Mycotoxins and Animal Foods* by Smith JE (Ed.). CRC-Press ISBN: 0-8493-4904-4

[172] Mehan VK, Bhavanishankar TN, Bedi JS (1985) Comparison of different methods for extraction and estimation of aflatoxin B_1 in groundnut. J Food Sci & Tech 22: 123-125

[173] Farag RS, El-leithy MA, Basyony AE, Daw ZY (1986) Effects of various substrates on aflatoxin production by *A. parasiticus*. JAOCS 63: 1024-1026

[174] Leibetseder J (1989) Die Bedeutung der Mycotoxine für Mensch und Tier. Ernährung 13: 739-745

[175] Tuinstra LGMTh, Haasnoot W (1983) Rapid determination of aflatoxin B_1 in dutch feeding stuffs by high-performance liquid chromatography and post-column derivatization. J Chrom 282: 47-462

[176] Marth EH (1990) Mycotoxins, in: *Foodborne Disease Handbook* by Cliver DO (Ed.) Academic Press ISBN: 0-121-76558-X

Chapter 5

Annex

5.1 Collaborative Trial Results on Food Stuffs

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.99	3.52	0.81	3.44	<0.2	<0.2
B	*0.00**	*0.50**	*0.10**	*0.40**	0	0
C	0.91	3.12	0.94	3.48	<0.01	0.05
D	*0.36**	3.77	*0.77**	4.00	<0.05	<0.05
E	0.80	4.03	0.80	3.87	<0.10	<0.10
F	0.74	2.93	0.73	2.91	<0.18	0.2
G	0.77	2.97	0.75	2.71	<0.52	<0.52
H	0.86	3.29	0.93	3.57	0.04	0.07
I	0.80	2.94	0.80	3.16	<0.1	<0.1
J	0.78	3.35	0.80	3.33	<0.15	<0.15
K	0.94	3.73	0.85	3.75	<0.18	<0.18
L	0.88	3.47	0.86	3.47	<0.01	<0.01
M	0.88	3.46	0.88	3.30	<0.1	<0.1
N	0.88	3.06	0.82	3.48	<0.1	<0.1
O	0.79	3.07	0.91	3.65	0.07	0.06
P	1.10	3.62	1.01	3.77	0.19	0.09
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	1.071	0.746	1.31	1.25	2.933	3.22
B	*0.2**	*0.1**	*0.2**	*0.3**	*0.3**	*0.4**
C	0.8	0.81	1.51	1.54	3.12	3.2
D	1.07	0.99	1.42	1.62	*3.84**	*1.61**
E	0.7	0.62	1.35	1.28	2.94	3.23
F	0.72	0.7	0.81	1.15	2.92	2.68
G	0.59	0.61	1.05	0.8	2.57	2.67
H	0.79	0.92	1.32	1.73	3.11	2.99
I	0.8	0.76	1.26	1.36	3.06	3.12
J	0.76	0.88	1.27	1.39	2.72	2.85
K	0.74	0.96	1.56	1.25	2.72	2.52
L	1.27	0.85	1.47	1.62	3.29	3.37
M	0.74	0.8	1.44	1.40	2.84	2.93
N	1.06	0.96	1.78	1.74	3.43	3.39
O	0.82	0.8	1.48	1.49	2.77	3.12
P	1.05	0.89	1.47	1.70	3.39	3.44

Table 5.1: Single results on aflatoxin B_1 in paprika powder

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.24	0.73	0.15	0.73	<0.1	<0.1
B	0.10	*0.30**	0.10	*0.20**	0	0
C	0.22	0.60	0.16	0.67	<0.01	<0.01
D	0.08	0.78	0.18	0.82	<0.05	<0.05
E	0.09	0.73	0.08	0.64	<0.04	<0.04
F	0.15	0.61	0.16	0.69	<0.04	<0.04
G	*<0.23**	0.59	*<0.23**	0.53	<0.23	<0.23
H	0.14	*0.30**	0.07	*0.64**	<*	<*
I	0.16	0.62	0.16	0.74	<0.05	<0.05
J	0.19	0.71	0.16	0.69	<0.07	<0.07
K	0.21	0.77	0.17	0.85	<0.06	<0.06
L	0.18	0.68	0.19	0.73	<0.01	<0.01
M	0.17	0.74	0.18	0.67	<0.08	<0.08
N	0.15	0.57	0.13	0.62	<0.1	<0.1
O	0.17	0.68	0.18	0.77	<0.02	<0.02
P	0.15	0.74	0.19	0.77	<0.03	<0.03
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	*<0.1**	*<0.1**	*0.125**	*<0.1**	0.205	0.176
B	0	0	0	0.1	*0.1**	*0.1**
C	0.05	0.04	0.14	0.08	0.2	0.19
D	0.07	0.1	0.1	0.16	*0.28**	*0.12**
E	*<0.04**	*<0.04**	*<0.04**	*<0.04**	*0.1**	*0.12**
F	0.06	0.05	0.07	0.09	0.19	0.2
G	*<0.23**	*<0.23**	*<0.23**	*<0.23**	*<0.23**	*<0.23**
H	0.04	0.06	0.09	0.1	0.17	0.18
I	0.06	0.06	0.1	0.1	0.2	0.22
J	0.04	0.03	0.09	0.08	0.2	0.21
K	0.061	0.078	0.149	0.102	0.225	0.188
L	0.07	0.05	0.08	0.1	0.19	0.2
M	*<0.08**	*<0.08**	0.12	0.15	0.2	0.22
N	*0.67**	*<0.1**	0.11	0.08	0.2	0.2
O	0.06	0.06	0.11	0.11	0.22	0.24
P	0.07	0.07	0.12	0.13	0.25	0.25

Table 5.2: Single results on aflatoxin B_2 in paprika powder

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.66	2.48	0.61	2.16	<0.2	<0.2
B	0.10	0.80	0.00	2.00	0	0
C	0.81	2.34	0.87	2.94	<0.01	<0.01
D	0.27	2.94	0.55	2.96	<0.05	<0.05
E	0.80	3.45	0.73	3.28	<0.20	<0.20
F	0.71	2.18	0.81	2.27	<0.3	<0.3
G	*<0.80**	2.40	*<0.80**	1.97	<0.80	<0.80
H	0.87	3.37	0.90	3.87	<	<
I	0.44	1.52	0.48	1.60	<0.1	<0.1
J	0.55	2.40	0.55	2.24	<0.20	<0.20
K	0.33	2.69	0.22	1.49	<0.2	<0.2
L	0.88	3.66	0.84	3.68	<0.01	<0.01
M	0.46	3.55	0.51	2.44	<0.05	<0.05
N	0.51	2.04	0.55	2.22	<0.1	<0.1
O	*0.36**	1.11	*0.87**	3.09	<0.02	<0.02
P	0.74	2.12	0.56	2.42	<0.06	<0.06
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	*<0.2**	*<0.2**	0.413	0.38	0.991	1.215
B	0.2	0.1	*0.8**	*0.7**	0.2	0.4
C	0.04	0.05	0.73	0.77	1.24	1.62
D	0.08	0.05	0.48	0.53	*1.34**	*0.56**
E	*<0.20**	*<0.20**	0.63	0.65	1.34	1.5
F	*<0.3**	*<0.3**	0.49	0.61	1.04	1.11
G	*<0.80**	*<0.80**	*<0.80**	*<0.80**	0.95	1.1
H	0.06	0.16	0.77	0.88	1.71	1.66
I	*<0.1**	*<0.1**	0.24	0.32	0.78	0.8
J	*<0.20**	*<0.20**	0.44	0.47	0.91	1.07
K	*<0.2**	*<0.2**	0.519	0.20	0.537	0.429
L	0.14	0.01	0.78	0.88	1.81	1.91
M	*<0.05**	*<0.05**	0.47	0.29	1.22	1.07
N	*<0.1**	*0.22**	0.64	0.43	0.98	0.97
O	0.1	0.09	0.55	0.48	1.16	1.29
P	0.15	0.13	0.62	0.62	1.48	1.54

Table 5.3: Single results on aflatoxin G_1 in paprika powder

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.15	0.55	0.18	0.69	<0.1	<0.1
B	0.10	0.40	0.10	0.40	0	0
C	*0.23**	0.40	*0.04**	0.54	<0.02	<0.02
D	0.08	0.62	0.15	0.70	<0.05	<0.05
E	0.19	0.63	0.10	0.65	<0.08	<0.08
F	0.14	0.50	0.18	0.57	<0.09	<0.09
G	0.17	0.58	0.16	0.44	<0.11	<0.11
H	0.09	0.08	0.09	0.39	<	<
I	0.10	0.36	0.12	0.40	<0.1	<0.1
J	0.12	0.59	0.13	0.57	<0.1	<0.1
K	*<0.15**	0.36	*<0.15**	0.58	<0.15	<0.15
L	0.13	0.44	0.09	0.48	<0.01	<0.01
M	*<0.10**	0.54	*<0.10**	0.46	<0.1	<0.1
N	0.14	0.48	0.12	0.50	<0.1	<0.1
O	*interf.**	*interf.**	*interf.**	*interf.**	*interf.**	*interf.**
P	0.18	0.49	0.15	0.55	<0.05	<0.05
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	<0.1	<0.1	<0.1	0.1	0.1	0.102
B	0	0	0	0	0	0.1
C	<0.02	<0.02	<0.02	<0.02	0.15	0.03
D	0.07	0.12	0.1	0.16	0.16	<0.05
E	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
F	<0.09	<0.09	<0.09	<0.09	0.11	0.13
G	<0.11	<0.11	<0.11	<0.11	0.13	0.13
H	<	<	<	0.06	0.06	0.04
I	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
J	<0.1	<0.1	<0.1	0.04	0.07	0.06
K	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15
L	<0.01	0.18	0.95	0.26	0.8	0.22
M	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
N	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O	*interf.**	*interf.**	*interf.**	*interf.**	*interf.**	*interf.**
P	<0.05	<0.05	<0.05	0.06	0.12	0.12

Table 5.4: Single results on aflatoxin G_2 in paprika powder

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	1.32	4.17	1.31	3.80	*0.352**	*<0.2**
B	1.30	3.50	1.30	3.20	0.6	0.4
C	*2.43**	*10.60**	*3.06**	*9.86**	0.2	0.15
D	1.30	4.50	0.62	4.25	0.25	0.27
E	0.60	3.06	1.03	2.97	0.29	0.33
F	0.94	3.54	1.13	3.33	0.34	0.35
G	1.04	3.10	0.92	3.46	*<0.53**	*<0.53**
H	1.11	3.42	1.02	3.67	0.36	0.33
I	1.17	3.96	1.34	3.93	0.36	0.36
J	1.20	3.70	1.17	3.50	0.39	0.2
K	1.38	4.45	1.15	3.47	0.344	0.309
L	1.19	3.49	1.19	3.43	0.19	0.19
M	0.70	3.61	0.83	3.41	0.36	0.37
N	1.11	4.00	1.14	3.42	0.44	0.34
O	0.90	3.35	1.19	4.13	0.4	0.36
P	1.32	3.77	0.96	2.31	0.46	0.17
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	1.421	1.333	2.334	2.14	3.297	2.939
B	1.7	1.7	2.2	2.30	3.1	2.7
C	0.67	0.75	*0.87**	*1.00**	1.4	1.15
D	1.57	1.28	2.33	2.43	3.39	3.26
E	1.09	1.09	1.41	1.41	1.9	0.6
F	1.03	1.22	1.88	1.56	1.94	2.38
G	0.93	0.69	1.83	1.71	1.22	2.62
H	1.67	1.47	*2.08**	*1.27**	1.69	2.33
I	1.59	1.56	2.37	2.31	3.12	3.33
J	1.43	1.5	2.25	2.20	3.04	2.99
K	1.375	1.058	1.557	2.000	2.125	2.508
L	1.53	1.43	2.24	2.15	3.33	2.98
M	1.42	1.33	2.11	2.04	2.8	2.42
N	1.47	1.49	1.99	2.13	2.82	3.35
O	1.25	0.89	2.06	2.12	2.65	2.51
P	1.66	1.55	2.47	2.39	2.75	2.86

Table 5.5: Single results on aflatoxin B_1 in fig paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.28	0.81	0.30	0.76	<0.1	<0.1
B	0.30	0.70	0.30	0.60	*0.1**	*0.1**
C	*0.51**	*2.12**	*0.62**	*2.01**	0.05	0.04
D	0.27	0.96	0.14	0.92	0.09	0.09
E	0.15	0.77	0.26	0.71	0.09	0.11
F	0.21	0.75	0.24	0.74	0.11	0.08
G	*<0.23**	0.66	*<0.23**	0.72	*<0.23**	*<0.23**
H	0.22	0.52	0.16	0.72	0.07	0.08
I	0.27	0.84	0.30	0.81	0.09	0.12
J	0.24	0.77	0.24	0.79	0.1	0.08
K	0.33	0.95	0.25	0.82	0.046	0.09
L	0.26	0.76	0.26	0.73	0.06	0.08
M	0.25	0.76	0.21	0.71	0.1	0.1
N	0.23	0.77	0.25	0.75	0.11	0.08
O	0.22	0.77	0.27	0.87	0.11	0.09
P	0.30	0.79	0.20	0.48	0.12	0.05
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.54	0.462	0.85	0.763	1.468	1.522
B	0.6	0.6	0.8	0.8	1.4	1.4
C	0.22	0.25	*0.3**	*0.34**	*0.63**	*0.53**
D	0.57	0.47	0.86	0.86	*1.52**	*1.55**
E	0.4	0.42	0.57	0.59	1.06	0.3
F	0.42	0.43	0.68	0.65	1.32	1.44
G	0.33	0.25	0.62	0.58	*0.56**	*1.14**
H	0.45	0.42	*0.6**	*0.39**	*0.69**	*0.84**
I	0.54	0.54	0.84	0.81	1.47	1.5
J	0.48	0.5	0.71	0.77	1.43	1.41
K	0.479	0.384	0.588	0.703	1.061	1.175
L	0.49	0.48	0.75	0.73	1.41	1.33
M	0.54	0.51	0.69	0.73	1.37	1.27
N	0.44	0.47	0.59	0.63	1.16	1.35
O	0.46	0.33	0.79	0.82	1.21	1.3
P	0.55	0.53	0.85	0.82	1.28	1.48

Table 5.6: Single results on aflatoxin B_2 in fig paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.81	3.29	0.82	3.04	<0.2	<0.2
B	*1.70**	2.60	*2.00**	3.70	1.1	1.1
C	*1.87**	*9.74**	*2.62**	*7.66**	0.08	0.08
D	0.89	3.63	0.41	3.62	<0.05	0.07
E	0.42	3.07	0.70	2.90	0.32	0.92
F	0.99	2.12	0.72	3.17	0.3	<0.3
G	*<0.83**	2.65	*<0.83**	2.89	<0.83	<0.83
H	1.05	3.31	0.91	3.81	0.24	0.22
I	0.96	3.48	1.02	3.24	0.21	0.21
J	0.60	2.41	0.56	2.17	<0.2	<0.2
K	0.50	1.07	0.84	1.07	0.137	<0.2
L	1.01	3.47	1.03	3.66	0.03	0.05
M	0.09	3.66	0.17	1.72	<0.05	<0.05
N	1.12	3.68	1.17	3.30	0.54	0.36
O	0.36	1.29	0.88	3.51	0.17	0.17
P	0.73	1.65	0.57	1.35	0.65	0.17
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.735	0.613	0.922	0.888	1.394	0.898
B	1.8	1.6	*1.6**	*2.5**	2	1.7
C	0.38	0.46	0.41	0.44	0.68	0.5
D	0.74	0.73	1.05	0.98	1.29	1.37
E	0.99	1.18	*0.31**	*1.55**	*1.24**	*0.29**
F	0.64	0.78	0.69	0.81	0.87	0.92
G	*<0.83**	*<0.83**	*<0.83**	*<0.83**	*<0.83**	*0.89**
H	1.28	1.03	*1.09**	*0.66**	0.85	1.1
I	0.9	0.87	0.99	1.05	1.32	1.35
J	0.84	0.96	0.92	0.79	0.93	0.92
K	0.381	0.511	*<0.2**	*0.245**	0.204	0.389
L	1	0.93	1.08	1.00	1.6	1.4
M	0.44	0.4	0.33	0.40	0.57	0.41
N	1.15	1.08	1.08	1.05	1.27	1.21
O	0.78	0.56	0.93	0.99	1.13	1.06
P	0.77	0.5	1.04	0.96	1.16	1.21

Table 5.7: Single results on aflatoxin G_1 in fig paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.15	0.63	0.25	0.69	<0.1	<0.1
B	0.30	0.50	0.40	0.70	0.2	0.3
C	0.49	*2.12**	0.53	*1.70**	<0.02	<0.02
D	*0.20**	0.78	*<0.05**	0.73	<0.05	<0.05
E	0.09	0.61	0.01	0.61	<0.08	<0.08
F	0.19	0.48	0.19	0.62	<0.09	<0.09
G	0.15	0.62	0.16	0.64	<0.10	<0.10
H	0.19	0.27	0.09	0.58	<0.15	<0.15
I	0.18	0.69	0.18	0.69	<0.1	<0.1
J	0.14	0.56	0.13	0.46	<0.1	<0.1
K	*<0.15**	0.28	*0.19**	0.27	<0.15	<0.15
L	0.17	0.72	0.17	0.67	0.02	<0.01
M	*<0.1**	0.69	*<0.1**	0.27	<0.1	<0.1
N	0.22	0.71	0.47	0.95	0.36	<0.1
O	0.10	0.35	0.20	0.80	<0.02	<0.02
P	0.14	0.40	0.11	0.30	<0.05	<0.05
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.22	0.232	0.406	0.378	0.726	0.551
B	0.5	0.4	0.5	0.60	0.8	0.7
C	0.08	0.12	0.14	0.16	0.27	0.17
D	0.26	0.29	0.35	0.43	0.68	0.62
E	0.2	0.24	0.31	0.27	0.46	0.22
F	0.18	0.22	0.31	0.30	0.46	0.56
G	0.17	0.11	0.25	0.23	0.25	0.43
H	0.27	0.22	0.3	0.20	0.3	0.34
I	0.27	0.27	0.39	0.39	0.63	0.63
J	0.26	0.26	0.37	0.35	0.52	0.58
K	0.149	0.212	*<0.15**	*<0.15**	*<0.15**	*0.242**
L	0.36	0.45	0.44	0.43	0.74	0.61
M	0.18	0.18	0.21	0.27	0.44	0.43
N	0.32	0.47	*0.55**	*0.28**	0.48	0.51
O	0.27	0.19	0.42	0.45	0.6	0.62
P	0.23	0.18	0.38	0.39	0.58	0.6

Table 5.8: Single results on aflatoxin G_2 in fig paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.86	3.55	0.95	3.67	*<0.2**	*<0.2**
B	*0.10**	*0.10**	*0.10**	*0.10**	0	0
C	0.85	3.57	0.97	3.40	0.14	0.1
D	1.09	*1.65**	1.32	*4.90**	*<0.05**	*0.12**
E	0.70	3.46	0.85	3.48	0.11	0.09
F	0.91	3.66	0.86	3.69	0.15	0.14
G	0.62	*1.85**	0.99	*2.67**	*<0.35**	*<0.35**
H	1.02	3.35	1.00	3.71	0.15	0.18
I	1.08	3.80	1.14	3.82	0.2	0.18
J	1.02	3.78	1.06	3.60	*0.16**	*0.39**
K	0.71	*1.64**	1.04	*?**	*<0.1**	*<0.1**
L	1.01	3.74	1.01	3.68	0.12	0.11
M	0.99	3.30	0.87	3.02	*<0.1**	*<0.1**
N	0.99	3.75	1.07	3.40	*0.2**	*0.55**
O	0.78	3.66	0.88	3.66	0.14	0.13
P	1.04	*4.56**	0.67	*1.63**	0.18	0.27
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.807	0.682	1.664	1.68	3.278	3.32
B	*0.1**	*0**	*0.1**	*0.2**	*0.2**	*0.2**
C	0.7	0.8	1.6	1.90	3.15	3.33
D	0.94	0.77	2.36	1.59	3.43	2.54
E	0.62	0.61	1.3	1.43	2.77	1.97
F	0.57	0.63	1.4	1.36	2.82	2.72
G	0.6	0.57	1.39	1.38	2.96	1.3
H	0.67	0.75	1.59	1.60	4.49	3.09
I	0.84	0.82	1.8	1.90	3.26	3.3
J	0.73	0.44	0.54	1.40	3.25	1.57
K	*0.256**	*0.244**	1.378	0.611	*0.826**	*0.759**
L	0.8	0.78	1.75	1.74	3.52	3.28
M	0.79	0.8	1.44	1.18	2.82	2.48
N	*0.17**	*0.73**	1.6	1.66	3.01	2.68
O	0.78	0.83	1.8	1.90	3.21	2.85
P	0.87	0.97	1.48	1.87	3.1	2.6

Table 5.9: Single results on aflatoxin B_1 in pistachio paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.15	0.73	0.20	0.77	<0.1	<0.1
B	*0.10**	*0.10**	*0.10**	*0.10**	0	0
C	0.18	0.83	0.20	0.80	0.02	<0.01
D	*0.26**	0.37	*0.24**	1.00	<0.05	<0.05
E	0.18	0.72	0.16	0.70	0.03	0.03
F	0.17	0.82	0.16	0.79	<0.03	<0.03
G	*<0.17**	0.40	*0.19**	0.58	<0.17	<0.17
H	0.18	0.47	0.17	0.72	<0.04	<0.04
I	0.20	0.76	0.22	0.76	<0.05	<0.05
J	0.18	0.73	0.20	0.72	<0.04	0.05
K	0.17	*0.34**	0.23	*?**	<0.04	<0.04
L	0.20	0.78	0.18	0.76	0.01	0.01
M	0.18	0.65	0.17	0.62	<0.08	<0.08
N	0.19	0.74	0.21	0.69	<0.1	<0.1
O	0.15	0.75	0.18	0.74	<0.02	<0.02
P	0.20	0.92	0.13	0.35	0.02	0.02
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	*0.103**	*<0.1**	0.163	0.159	0.339	0.335
B	*0**	*0**	0.1	0.1	*0.1**	*0.1**
C	0.08	0.08	0.15	0.2	0.32	0.35
D	0.13	0.11	0.22	0.17	0.35	0.26
E	0.1	0.09	0.14	0.15	0.29	0.22
F	0.08	0.09	0.17	0.12	0.17	0.26
G	*<0.17**	*<0.17**	*<0.17**	*<0.17**	*0.28**	*<0.17**
H	0.09	0.09	0.14	0.15	0.43	0.27
I	0.12	0.1	0.18	0.18	0.32	0.34
J	0.09	0.06	0.05	0.12	0.32	0.18
K	0.041	0.048	0.177	0.09	*0.097**	*0.106**
L	0.1	0.1	0.14	0.17	0.32	0.31
M	0.11	0.11	0.16	0.14	0.31	0.27
N	*<0.1**	*0.11**	0.15	0.16	0.28	0.23
O	0.1	0.11	0.18	0.2	0.34	0.3
P	0.11	0.12	0.17	0.19	0.33	0.28

Table 5.10: Single results on aflatoxin B_2 in pistachio paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.64	3.05	0.85	3.47	<0.2	<0.2
B	0.20	*0.30**	0.00	*0.10**	0	0
C	0.66	2.87	0.76	2.58	<0.01	<0.01
D	1.10	1.69	0.93	4.07	<0.05	<0.05
E	0.76	3.51	0.69	3.28	<0.20	<0.20
F	0.75	3.66	0.76	3.23	<0.2	<0.2
G	*<0.53**	1.92	*0.77**	2.51	<0.53	<0.53
H	0.92	3.22	0.91	3.67	<0.1	<0.1
I	0.94	3.60	0.94	3.50	<0.1	<0.1
J	0.79	3.42	0.88	3.28	<0.15	<0.15
K	0.34	*1.21**	0.66	*?**	<0.13	<0.13
L	0.97	3.87	0.92	3.81	<0.01	<0.01
M	0.43	2.69	0.31	1.83	<0.2	<0.2
N	0.76	2.89	0.77	2.52	<0.1	<0.1
O	0.58	2.93	0.83	3.87	<0.02	<0.02
P	0.68	3.25	0.47	1.17	<0.04	<0.04
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
B	0	0	0	0	0	0
C	<0.01	<0.01	<0.01	<0.01	<0.01	0.08
D	<0.05	<0.05	<0.05	<0.05	0.07	<0.05
E	<0.20	<0.20	<0.20	<0.20	<0.20	<0.20
F	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
G	<0.53	<0.53	<0.53	<0.53	<0.53	<0.53
H	<0.1	<0.1	<0.1	0.05	0.18	0.12
I	<0.1	<0.1	0.1	0.1	0.14	0.14
J	<0.15	<0.15	<0.15	<0.15	<0.15	<0.15
K	<0.13	<0.13	<0.13	<0.13	<0.13	<0.13
L	<0.01	<0.01	0.05	0.07	0.17	0.17
M	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
N	<0.1	<0.1	0.13	<0.1	<0.1	0.11
O	<0.02	<0.02	0.07	0.09	0.12	0.11
P	0.08	0.2	0.1	0.22	0.15	0.24

Table 5.11: Single results on aflatoxin G_1 in pistachio paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.13	0.61	0.15	0.73	<0.1	<0.1
B	0.20	0.30	0.10	0.20	0	0
C	0.15	0.70	0.20	0.60	<0.02	<0.02
D	0.24	0.35	0.18	0.87	<0.05	<0.05
E	0.17	0.74	0.15	0.66	<0.08	<0.08
F	0.17	0.78	0.17	0.63	<0.09	<0.09
G	0.11	0.43	0.18	0.58	<0.08	<0.08
H	0.17	0.27	0.09	0.66	<0.1	<0.1
I	0.20	0.72	0.20	0.74	<0.1	<0.1
J	0.18	0.68	0.19	0.70	<0.08	<0.08
K	0.11	*0.25**	0.13	*?**	<0.1	<0.1
L	0.19	0.76	0.17	0.77	<0.01	<0.01
M	*0.15**	0.60	*<0.10**	0.43	<0.1	<0.1
N	0.19	0.67	0.21	0.58	<0.1	<0.1
O	0.12	0.64	0.17	0.80	<0.02	<0.02
P	0.13	0.71	0.09	0.25	<0.03	<0.03
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
B	0	0	0	0	0	0
C	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
D	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
E	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
F	<0.09	<0.09	<0.09	<0.09	<0.09	<0.09
G	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
H	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
I	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
J	<0.08	<0.08	<0.08	<0.08	<0.08	<0.08
K	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
L	<0.01	<0.01	<0.01	<0.01	<0.01	0.015
M	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
N	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
O	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02
P	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03

Table 5.12: Single results on aflatoxin G_2 in pistachio paste

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.97	3.68	0.84	3.54	<0.2	<0.2
B	*0.90**	*3.60**	*0.10**	*0.40**	0	0
C	1.40	5.05	1.06	5.38	<0.01	<0.01
D	0.94	4.46	1.13	4.52	<0.05	<0.05
E	0.68	2.75	0.62	2.44	<0.10	<0.10
F	0.78	*3.05**	0.69	*1.58**	<0.12	<0.12
G	0.85	3.06	0.69	3.12	<0.34	<0.34
H	0.80	3.27	0.78	3.24	<0.1	<0.1
I	0.92	3.50	0.92	3.62	<0.1	<0.1
J	0.93	3.58	0.87	3.72	<0.1	<0.1
K	0.82	*3.13**	0.71	*2.39**	<0.1	<0.1
L	0.86	3.57	0.86	3.58	<0.01	<0.01
M	0.80	3.00	0.76	3.19	<0.10	¡
N	0.91	3.67	0.85	3.73	<0.1	<0.1
O	0.80	3.46	0.81	3.59	<0.02	<0.02
P	1.11	4.03	1.08	4.06	0.03	0.13
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.8527	0.929	1.37	1.54	3.206	3.559
B	*0.2**	*0.2**	*0.3**	*0.2**	*1.2**	*0.1**
C	1.02	1.04	1.59	1.65	3.9	4
D	0.96	1.07	1.8	1.50	3.83	3.86
E	0.65	0.56	1.18	1.21	2.69	2.71
F	0.7	0.76	1.14	1.05	2.79	2.84
G	0.68	0.62	1.22	1.34	2.79	3.08
H	0.87	0.84	1.49	1.55	3.44	3.55
I	0.98	0.98	1.58	1.76	4	3.96
J	*0.96**	*0.53**	1.71	1.70	3.99	3.76
K	0.419	0.303	*1.643**	*4.830**	*0.468**	*0.588**
L	1.02	0.87	1.8	1.80	4	4.26
M	0.85	0.79	1.52	1.54	3.31	3.2
N	0.89	0.88	1.68	1.42	1.69	2.02
O	0.88	0.91	1.45	1.49	3.49	3.37
P	1.14	1.13	1.76	1.65	3.93	4.02

Table 5.13: Single results on aflatoxin B_1 in peanut butter

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.20	0.74	0.20	0.74	<0.1	<0.1
B	0.20	*0.80**	0.10	*0.30**	0	0
C	0.29	1.04	0.22	1.11	<0.01	<0.01
D	0.19	0.91	0.25	0.94	<0.05	<0.05
E	0.19	0.63	0.18	0.66	<0.04	<0.04
F	0.18	*0.75**	0.16	*0.37**	<0.04	<0.04
G	*0.17**	0.65	*<0.16**	0.67	<0.16	<0.16
H	0.15	0.45	0.11	0.61	<0.04	<0.04
I	0.20	0.72	0.20	0.74	<0.05	<0.05
J	0.19	0.75	0.17	0.79	<0.04	<0.04
K	0.19	0.71	0.18	0.56	<0.05	<0.05
L	0.19	0.75	0.19	0.74	<0.01	<0.01
M	0.19	0.62	0.18	0.64	<0.07	<0.07
N	0.17	0.71	0.15	0.76	<0.1	<0.1
O	0.17	0.74	0.18	0.77	<0.02	<0.02
P	0.23	0.84	0.21	0.85	<0.02	<0.02
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.148	0.152	0.27	0.334	0.719	0.769
B	0.1	0.1	*0.1**	*0.1**	*0.4**	*0.2**
C	0.17	0.18	0.32	0.33	0.8	0.81
D	0.18	0.19	0.38	0.3	0.81	0.81
E	0.18	0.14	*0.28**	*1.31**	0.65	0.69
F	0.14	0.15	0.31	0.3	0.6	0.67
G	*<0.16**	*<0.16**	0.25	0.26	0.61	0.63
H	0.13	0.13	0.27	0.28	0.6	0.64
I	0.18	0.18	0.34	0.36	0.84	0.84
J	*0.19**	*0.09**	0.37	0.36	0.85	0.81
K	0.094	0.069	*0.358**	*0.137**	*0.121**	*0.128**
L	0.18	0.16	0.33	0.34	0.75	0.81
M	0.16	0.16	0.33	0.32	0.72	0.71
N	0.14	0.15	0.28	0.23	0.38	0.44
O	0.16	0.16	0.32	0.33	0.73	0.73
P	0.19	0.19	0.37	0.34	0.84	0.87

Table 5.14: Single results on aflatoxin B_2 in peanut butter

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.82	3.48	0.81	3.38	<0.2	<0.2
B	*2.00**	*7.90**	*0.20**	*1.20**	0	0
C	1.08	4.40	0.80	4.16	<0.01	<0.01
D	0.68	3.61	0.91	4.09	<0.05	<0.05
E	0.67	2.91	0.68	3.44	<0.20	<0.20
F	0.72	2.79	0.68	2.24	<0.3	<0.3
G	0.74	2.92	0.55	2.83	<0.49	<0.49
H	0.80	3.20	0.78	3.28	<0.1	<0.1
I	0.86	3.22	0.80	3.16	<0.1	<0.1
J	0.80	3.12	0.72	3.19	<0.15	<0.15
K	0.82	*1.51**	0.41	*0.89**	<0.13	<0.13
L	0.92	3.84	0.92	3.88	<0.01	<0.01
M	0.64	*0.69**	0.33	*2.14**	<0.2	<0.2
N	0.81	3.19	0.73	3.39	<0.1	<0.1
O	0.74	3.16	0.85	3.75	<0.02	<0.02
P	0.77	2.81	0.82	3.07	<0.04	<0.04
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.31	0.355	0.316	0.289	0.614	0.797
B	0.2	0.1	*0.1**	*0.1**	0.6	0
C	0.44	0.45	0.32	0.34	0.81	0.7
D	0.34	0.33	0.27	0.2	0.69	0.65
E	0.37	0.32	0.41	0.41	0.7	0.73
F	0.42	0.47	0.39	0.27	0.6	0.61
G	*<0.49**	*<0.49**	*<0.49**	*<0.49**	*<0.49**	*<0.49**
H	0.42	0.4	0.34	0.36	0.77	0.79
I	0.36	0.38	0.32	0.36	0.8	0.82
J	0.29	0.18	0.35	0.32	0.76	0.71
K	*<0.13**	*<0.13**	*0.368**	*<0.13**	*<0.13**	*<0.13**
L	0.51	0.48	0.44	0.43	0.96	1.04
M	*<0.20**	*<0.2**	0.33	0.4	0.48	0.3
N	0.36	0.4	0.36	0.3	0.4	0.49
O	0.43	0.38	0.32	0.33	0.7	0.74
P	0.58	0.48	0.3	0.39	0.87	0.89

Table 5.15: Single results on aflatoxin G_1 in peanut butter

Lab ID	**spike (a1)**	**spike (b1)**	**spike (a2)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
A	0.14	0.68	0.16	0.70	<0.1	<0.1
B	*0.40**	*1.40**	*0.20**	*0.50**	0	0
C	*0.24**	0.88	*0.10**	0.77	<0.02	<0.02
D	0.15	0.78	0.22	0.84	<0.05	<0.05
E	0.15	0.69	0.13	0.71	<0.08	<0.08
F	0.14	0.36	0.16	0.46	<0.09	<0.09
G	0.17	0.69	0.14	0.67	<0.07	<0.07
H	*0.13**	0.17	*0.03**	0.50	<0.1	<0.1
I	0.18	0.66	0.16	0.68	<0.1	<0.1
J	0.17	0.69	0.15	0.66	<0.08	<0.08
K	*0.17**	0.34	*<0.1**	0.19	<0.1	<0.1
L	0.16	0.60	0.16	0.59	<0.01	<0.01
M	*<0.05**	0.18	*<0.05**	0.44	<0.05	<0.05
N	0.15	0.70	0.15	0.75	<0.1	<0.1
O	0.16	0.70	0.19	0.80	<0.02	<0.02
P	0.17	0.64	0.18	0.68	<0.03	<0.03
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
A	0.102	0.101	*<0.1**	*0.115**	0.185	0.234
B	*0.2**	*0.1**	*0.1**	*0.2**	0.3	0.2
C	0.11	0.1	0.05	0.07	0.22	0.16
D	0.080	0.100	0.09	0.06	0.21	0.23
E	0.12	0.09	0.1	0.13	0.23	0.2
F	0.12	0.13	0.13	0.08	*0.18**	*0.49**
G	0.07	0.07	*<0.07**	*<0.07**	0.16	0.17
H	0.09	0.07	0.07	0.07	0.17	0.17
I	0.1	0.1	0.1	0.1	0.22	0.24
J	*0.08**	*<0.08**	0.08	0.1	0.21	0.23
K	*<0.1**	*<0.1**	*0.103**	*<0.1**	*<0.1**	*<0.1**
L	0.09	0.12	0.08	0.1	0.27	0.28
M	*<0.05**	*<0.05**	*0.06**	*<0.05**	*0.18**	*<0.05**
N	0.11	0.11	0.1	0.095	0.13	0.14
O	0.1	0.11	0.1	0.1	0.18	0.23
P	0.13	0.13	0.09	0.09	0.25	0.27

Table 5.16: Single results on aflatoxin G_2 in peanut butter

5.2 Collaborative Trial Results on Infant Formula

Lab ID	**spike (a1)**	**spike (a2)**	**spike (b1)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
1	0.09	0.09	0.18	0.17	<0.02	<0.02
2	0.12	0.12	0.22	0.23	0.02	0.02
3	*0.11**	*0.19**	0.10	0.19	0.02	0.01
4	0.11	0.11	0.19	0.20	<0.01	<0.01
5	0.11	0.10	0.20	0.22	<0.01	<0.01
6	0.11	0.11	0.20	0.20	<0.02	<0.02
7	0.09	0.08	0.18	0.17	<0.02	<0.02
8	0.09	0.09	0.17	0.16	<0.02	<0.02
9	*0.03**	*0.04**	0.11	0.06	0.02	0.02
10	0.09	0.10	0.19	0.21	<0.04	<0.04
11	0.10	0.11	0.20	0.20	0.01	0.01
12	0.11	0.10	0.27	0.21	<0.02	<0.02
13	0.07	0.10	0.20	0.20	0.03	0.02
14	0.09	0.09	0.16	0.15	0.01	0.01
Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
1	0.05	0.05	0.08	0.07	0.19	0.16
2	0.09	0.10	*0.11**	*0.12**	0.25	0.21
3	0.08	0.08	0.09	0.09	0.21	0.17
4	0.11	0.08	*0.13**	*0.09**	0.17	0.21
5	0.07	0.08	0.10	0.09	0.18	0.23
6	0.07	0.08	0.10	0.09	0.18	0.19
7	0.07	0.07	0.10	0.09	0.18	0.19
8	0.07	0.06	0.08	0.07	0.15	0.15
9	0.05	0.02	*0.06**	*0.05**	0.08	0.10
10	0.08	0.07	0.08	0.09	0.15	0.15
11	0.07	0.07	0.09	0.09	0.21	0.17
12	0.07	0.07	0.08	0.09	*0.39**	*0.18**
13	0.09	0.07	0.09	0.07	0.15	0.19
14	0.07	0.05	0.08	0.08	0.13	0.12

Table 5.17: Single results on aflatoxin B_1 in infant formula

5.3 Collaborative Trial Results on Animal Feed

Lab ID	**spike (a1)**	**spike (a2)**	**spike (b1)**	**spike (b2)**	**nc (c1)**	**nc (c2)**
1	1.26	1.42	3.94	4.10	<0,2	<0,2
2	1.17	0.90	3.62	3.62	0.00	0.14
3	1.59	1.47	4.48	4.70	<0.34	<0.34
4	1.28	1.31	3.77	3.76	0.04	0.05
5	0.92	1.08	2.97	2.88	0.12	0.08
6	1.33	1.13	3.70	3.60	<0,2	<0,2
7	1.13	1.11	3.50	3.36	<0,25	<0,25
8	1.47	1.46	3.89	3.95	0.16	0.12
9	1.63	1.63	4.88	4.20	0.11	0.14
10	1.10	1.04	3.64	2.77	0.00	0.00
11	1.44	1.41	4.33	4.31	0.54	0.62
12	1.49	1.44	4.29	4.38	0.08	0.01
13	1.01	1.01	2.99	2.77	0.00	0.01
14	0.94	0.92	2.80	2.67	n.d.	n.d.
15	1.88	1.70	4.46	3.56	1.11	0.80
16	1.28	1.21	3.75	3.67	0.04	0.07
17	1.52	1.42	3.46	3.75	0.10	<0.05
18	1.38	1.45	4.01	4.02	0.12	0.08
19	1.27	1.32	3.66	3.76	0.05	0.05
20	1.80	1.75	5.60	5.30	0.12	0.10
21	*5.44**	*5.60**	*15.84**	*14.32**	0.96	0.80

Table 5.18: Single results on aflatoxin B_1 in animal feed (Part1)

Lab ID	**nc (d1)**	**nc (d2)**	**nc (e1)**	**nc (e2)**	**nc (f1)**	**nc (f2)**
1	0.67	0.61	1.00	0.96	4.70	5.04
2	0.56	0.51	0.71	0.86	3.93	4.29
3	0.62	0.63	1.03	0.75	4.34	3.86
4	0.56	0.49	0.90	0.79	4.85	5.06
5	0.46	0.46	0.78	0.72	3.02	3.10
6	0.59	0.61	0.89	1.14	5.98	5.40
7	0.49	0.46	0.79	0.73	4.18	4.26
8	0.59	0.66	0.96	0.90	4.84	4.19
9	0.64	0.72	1.07	1.02	*18.36**	*14.72**
10	0.40	0.49	0.72	0.72	4.10	4.12
11	0.92	0.95	5.67	5.04	0.03	0.02
12	0.57	0.52	0.94	0.97	4.93	4.70
13	0.35	0.35	0.68	0.65	3.48	3.25
14	0.45	0.40	0.59	0.67	2.93	3.12
15	*0.99**	*0.97**	1.26	1.26	3.90	4.43
16	0.44	0.47	0.70	0.75	3.84	4.54
17	0.50	0.60	0.75	0.85	3.65	3.95
18	0.51	0.46	0.82	0.82	3.33	3.58
19	0.56	0.54	0.90	0.83	3.31	3.34
20	0.65	0.70	1.13	1.06	5.60	5.83
21	*3.20**	*2.48**	*4.32**	*4.16**	*21.84**	*16.56**

Table 5.19: Single results on aflatoxin B_1 in animal feed (Part2)